Universidade de Aveiro Departamento de Biologia
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Maria João Teixeira Ribeiro de Magalhães

Projecções encefálicas do núcleo reticular ventral do bolbo raquidiano

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Armando Alberto Nova Pinto Almeida, Professor Associado, Escola de Ciências da Saúde da Universidade do Minho, e co-orientação do Professor Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar, Departamento de Biologia da Universidade de Aveiro.

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Palavras-chave

Sistema nervoso; formação reticular; vias da dor; subunidade B da toxina da cólera; dextrano-amina biotinilado; projecções eferentes encefálicas.

Resumo

O Sistema Nervoso (SN) dos mamíferos é uma rede complexa de células especializadas na recepção, transmissão e integração de informação. O desempenho de cada um dos subsistemas depende da forma como a comunicação entre as diferentes áreas se organiza. Várias ferramentas têm vindo a ser desenvolvidas para o efeito, sendo actualmente os traçadores neuroanatómicos aquelas de uso mais abrangente. Após injecção do traçador seleccionado na área pretendida do SN, este é incorporado e subsequentemente transportado de forma retrógrada ou anterógrada de acordo com as suas propriedades. O estudo dos sistemas supraespinais de controlo da dor tem em muito beneficiado do uso desta tecnologia. Várias áreas do encéfalo e, em particular, da formação reticular do tronco cerebral, participam na modulação supraspinal da dor. O núcleo reticular ventral (VRt) do bolbo raquidiano continua a ser uma área pouco explorada do encéfalo, contrariamente ao seu homólogo dorsal (DRt), cujo envolvimento na modulação da dor se encontra bem estabelecido. No presente trabalho, as projecções encefálicas (eferentes e aferentes) do VRt são analisadas no rato, recorrendo-se para tal a injecções intracerebrais de traçadores neuronais anterógrados e retrógrados, respectivamente o dextrano-amina biotinilado (BDA) e a subunidade B da toxina da cólera (CTb). Verificou-se que os neurónios do VRt recebem projecções e projectam para áreas do encéfalo implicadas no processamento somatosensitivo, emocional e cognitivo da dor. Estes resultados corroboram com o papel do VRt na modulação da dor. As projecções encefálicas do VRt e DRt para o tronco cerebral são em si muito semelhantes, com o VRt a projectar para áreas mais restritas do diencéfalo. O papel de cada um dos núcleos na modulação da dor poderá estar relacionado com as diferenças observadas nas projecções dos núcleos.

Keywords Nervous system; medullary reticular formation; pain pathways; cholera toxin subunit B; biotinylated dextran; brain efferents.

Abstract

The nervous system (SN) of a mammal is a complex network of cells specialized for the reception, transmission and integration of information. The performance of each subsystem depends on how the communication between different areas is organized. Several tools have been developed for this purpose, being currently the neuroanatomical tracers those of wider use. After selected tracer injection in the desired area of the SN, this one is incorporated and subsequently transported anterogradely and retrogradely according to their properties. The study of the supraspinal pain control systems has greatly benefited from the use of this technology. Several areas of the brain and, in particular, the reticular formation of the brainstem, are involved in supraspinal pain modulation. The ventral portion of the caudal reticular formation (VRt) remains a relatively unexplored area of the brain contrary to its dorsal counterpart (DRt), whose involvement in pain modulation is well established. In the present work, the VRt brain connections (efferent and afferent projections) are investigated in the rat, using iontophoretic injections of the anterograde tracer biotinylated-dextran amine (BDA) and the retrograde tracer cholera toxin-subunit (CTb). It was found that neurons from the VRt receive and project to areas of the brain involved in somatosensitive, emotional and cognitive pain processing.

The set of brain projections observed in VRt is compatible with a role in pain modulation. VRt and DRt brain projections to the brainstem are similar; however, concerning to the diencephalon, VRt has a narrower set of targets. It remains unclear how these differences relate to differential roles in pain modulation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Index of Abbreviations

The abbreviations are listed in alphabetical order. Each abbreviation is followed by the structure name. The nomenclature and abbreviations used to designate brain nuclei and fiber tracts are in accordance with those used by Paxinos and Watson (1998) or result from a simplification of it, except for a few exceptions assigned with (*).

3	layer 3 of cortex
3V	3rd ventricle
7	facial nucleus
10N	dorsal motor nucleus of vagus
12N	hypoglossal nucleus
A	
A5/A7	noradrenaline cells
ABC	avidin–biotin complex
Amb	ambiguous nucleus
Amy	amygdaloid nucleus
AP	area postrema
APT	anterior pretectal nucleus
Aq	aqueduct
Arc	arcuate hypothalamic nucleus
B	
BDA	biotinylated dextran
BST	bed nucleus of the stria terminalis
BSTLV	bed nucleus of the stria terminalis, lateral division, ventral part
BSTMA	bed nucleus of the stria terminalis, medial division, anterior part
C	
CC	central canal
CeC	central amygdaloid nucleus, capsular part
CeCv	central cervical nucleus of the spinal cord
CeL	central amygdaloid nucleus, lateral division
CeM	central amygdaloid nucleus, medial division
CG	central gray
CL	centrolateral thalamic nucleus
CM	central medial thalamic nucleus
CnF	cuneiform nucleus
CTb	cholera toxin subunit B
Cu	cuneate nucleus
D	
DCDp	dorsal cochlear nucleus, deep core
DK	nucleus of Darkschewitsch
DLPAG	dorsolateral periaqueductal gray
DPGi	dorsal paragigantocellular nucleus
DpMe	deep mesencephalic nucleus
DR	dorsal raphe nucleus
DRt*	dorsal reticular nucleus
E	
ECu	external cuneate nucleus
Eth	ethmoid thalamic nucleus
F	
f	fornix

G

Gi	gigantocellular reticular nucleus
Giα	gigantocellular reticular nucleus alpha part
GiV	gigantocellular reticular nucleus ventral part
GP	globus pallidus
Gr	gracile nucleus

H

HDB	nucleus of the horizontal limb of the diagonal band

I

IG	indusium griseum
IMLF	interstitial nucleus of the medial longitudinal fasciculus
IO/IOM	inferior olive/medial nucleus
IP	interpeduncular nucleus
IRt	intermediate reticular nucleus

K

KF	Kölliker-Fuse nucleus

L

LC	locus coeruleus
LH	lateral hypothalamus
LL	nuclei of the lateral lemniscus
LM	lateral mammillary nucleus
LPGi	lateral paragigantocellular nucleus
LPO	lateral preoptic nucleus
LRt	lateral reticular nucleus
LS/LSI	lateral septal nuclei/intermediate part
ltg	lateral tegmental tract

M

Me5	mesencephalic trigeminal nucleus
mlf	medial longitudinal fasciculus
MnA	median accessory nucleus of the medulla
Mo5	motor trigeminal nucleus
MPO	medial preoptic nucleus
MS	medial septal nucleus

N

NTS*	nucleus tractus solitarius

P

PAG	periaqueductal gray
PB	parabrachial nuclei
PBS	saline phosphate buffer
PBS-T	0.1 M saline phosphate buffer containing 0.3% Triton X-100
PC	paracentral thalamic nucleus
PCRt	parvicellular reticular nucleus
Pe	periventricular hypothalamic nucleus
PF	parafascicular thalamic nucleus
PH	posterior hypothalamic area
PMn	paramedian reticular nucleus
Pn	pontine nuclei
PnC	pontine reticular nucleus, caudal part
PnO	pontine reticular nucleus, oral part
PnV	pontine reticular nucleus, ventral part
Po	posterior thalamic nuclear group
Pr	prepositus nucleus
PV	paraventricular thalamic nucleus
PVN*	paraventricular hypothalamic nucleus
py	pyramidal tract

R

R	red nucleus
Re	reunions thalamic nucleus
Rh	rhomboid thalamic nucleus
RIP	raphe interpositus nucleus
RMg	raphe magnus nucleus
RPa	raphe pallidus nucleus
ROb	raphe obscurus nucleus
Rt	reticular thalamic nucleus
RVM	rostral ventromedial medulla

S

SGe	supragenual nucleus
SHi	septohippocampal nucleus
SNC	substantia nigra, compact part
SNR	substantia nigra, reticular part
SolC	nucleus of the solitary tract, commissural part
SolM	nucleus of the solitary tract, medial part
SolV	nucleus of the solitary tract, ventral part
SolVL	nucleus of the solitary tract, ventrolateral part
sp5	spinal trigeminal nucleus
Sp5C	spinal trigeminal nucleus, caudal part
SPF	subparafascicular thalamic nucleus
SpVe	spinal vestibular nucleus
SubC	subcoeruleus nucleus

V

VA	ventral anterior thalamic nucleus
VDB	nucleus of the vertical limb of the diagonal band
VL	ventrolateral thalamic nucleus
VLH	ventrolateral hypothalamic nucleus
VLMlat*	lateral portion of the caudal ventrolateral medulla
VM	ventromedial thalamic nucleus
VMH	ventromedial hypothalamic nucleus
VP	ventral pallidum
VPL	ventral posterolateral thalamic nucleus
VPM	ventral posteromedial thalamic nucleus
VRt*	ventral reticular nucleus
VTA	ventral tegmental area

X

X	nucleus X

Chapter 1

INTRODUCTION

1.1 – Overview of the Nervous System

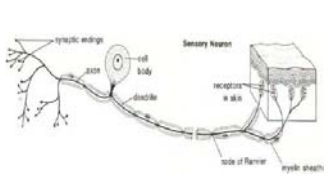
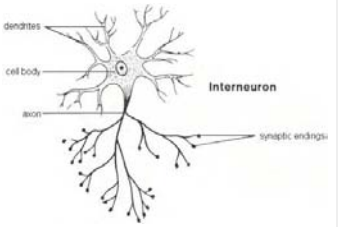
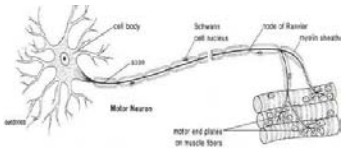
What distinguishes the mammals from other animals is the possession of a more or less elaborate system for rapidly receiving, integrating and transferring information through the body in the form of electrical signals, or nervous impulses. Anatomically, the nervous system is divided in the central nervous system (CNS) that comprises the brain and spinal cord, and the peripheral nervous system (PNS), where afferent sensory nerves transmit information to the CNS, while efferent motor nerves convey instructions from it. The fundamental units of the nervous system are the neurons, which working together form complex and organized networks for communication and information processing. In addition to neurons, there are glial cells that play a supporting role (Kandel et al., 1991) and can even modify communication between neurons (Auld and Robitaille, 2003).

1.1.1 Cellular Elements of Nervous System

The nervous system is made up of more than 100 billion nerve cells. These cells are classified as either neurons or glial cells, each of which has several sizes and shapes.

Neurons are specialized secretory cells composed of a cell body (soma, perikaryon), dendrites and an axon (Zigmond et al., 1999). Neurons usually receive messages from other neurons through the dendrites that pick up messages and carry them to the neuron's cell body. The axon carries outgoing messages from the cell. Some axons are covered with a myelin sheath, made up of glial cells, the Schwann cells at the periphery and oligodendrocytes centrally. This asymmetric organization allows the neurons to send and receive electrochemical signals and release of signaling molecules. As described in Table 1, according to their function within the nervous system, neurons can be divided into: a) sensory (afferent) neurons - cells that carry messages from the sense organs to the brain or spinal cord, b) motor (efferent) neurons - cells that carry messages from the brain or spinal cord to the muscles and glands, and c) interneurons - association neurons carrying messages from one neuron to another.

Table 1 – The different types of neurons found in the nervous system.

	Sensory neuron	Interneuron	Motor Neuron
Length of Fibers	Long dendrites and short axon.	Short dendrites and short or long axon.	Short dendrites and long axons.
Location	Cell body and dendrite are outside of the spinal cord; the cell body is located in a dorsal root ganglion.	Interneuron cell bodies are always located in the central nervous system (CNS).	Dendrites and the cell body are located in the spinal cord; the axon is outside of the spinal cord.
Function	Conduct impulse to the spinal cord.	Interconnect the sensory neuron with an appropriate motor neuron.	Conduct impulse to an effector (muscle or gland).
Example			

The three types of neurons are arranged in circuits and networks, the simplest of which is the reflex arc. In a simple reflex arc, such as the knee jerk, a stimulus is detected by a receptor cell, which is located in the peripheral branch of a sensory neuron. The sensory neuron carries the impulse from site of the stimulus to the CNS, where it synapses with an interneuron. The interneuron synapses with a motor neuron, which carries the nerve impulse out to an effector, such as a muscle, which responds by contracting.

Glial cells far outnumber neurons, comprises the other major cellular constituent of the nervous system. During many years it was thought that functionally, these cells were only involved in support, protection and nutrition of neurons, and also to have a facilitatory role in conduction for the neurons they surround (Auld and Robitaille, 2003; Ndubaku and Bellard, 2008). Lately, it has been shown that these cells have other functions: they participate in synaptic transmission and modulation, as key regulators of neurotransmitter release, and also as instructors for the development, maintenance, and recovery of synapses (Fields and Stevens-Graham, 2002; Auld and Robitaille, 2003; Allen and Barres, 2009). Glial cells have also been implicated in other neuron-glial interactions that contribute to glial proliferation, differentiation, myelination, among others (Auld and Robitaille, 2003; for reviews, see Barres and Raff, 1999; Fields and Stevens-Graham, 2002).

There are three types of glial cells in the CNS – astrocytes, oligodendrocytes and microglia – and one type in the PNS – Schwann cells – that fill up the spaces between neurons with layers of myelin membrane around axons to insulate them for impulse conduction (Figure 1) (Kandel et al., 1991; Fields and Stevens-Graham, 2002).

Astrocytes are known by their star-like shape and by the extensive end-feet on their processes. They perform many functions, including biochemical support of endothelial cells which form the blood-brain barrier, the provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a principal role in the repair and scarring process of the brain and spinal cord following traumatic injuries. Microglia are nonneuronal cells found in the brain that respond to injury or disease by surrounding cellular "trash" and activating inflammatory responses. Recent studies have shown that microglia can respond to neural impulse activity, thus mediating neuroimmune interactions, i.e. in chronic pain conditions (Watkinis et al., 2001; Brodal, 2004). Oligodendrocytes can be distinguished from astrocytes by having less and thinner processes. They form myelin sheaths around axons in the CNS, by enveloping them with concentric layers of plasma membrane. In PNS, these functions are performed by Schwann cells, forming myelin around PNS axons, ensheathing synaptic junctions, and bundling small-diameter axons together (Fields and Stevens-Graham, 2002). Myelin forms an insulating sheath around an axon, leaving small areas of axonal membrane exposed between successive myelin segments called nodes of Ranvier (Figure 2) (Watkinis et al., 2001; Poliak and Peles, 2003).

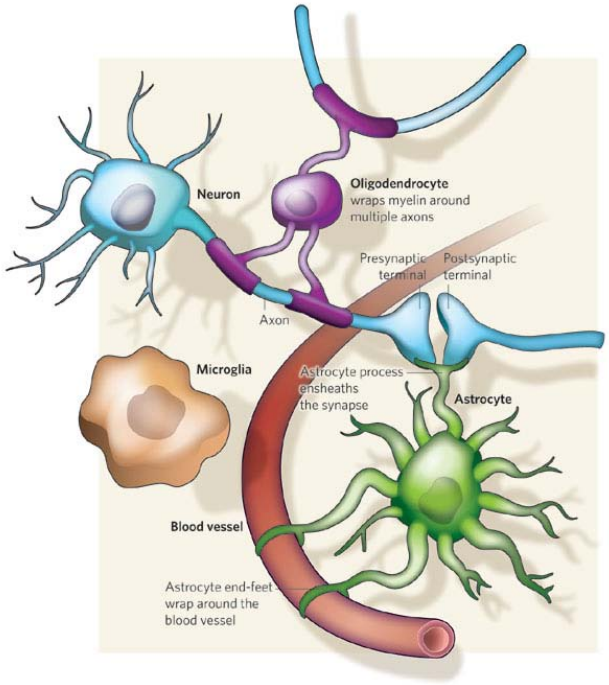


Figure 1 – *Glia-neuron interactions*. Different types of glia interact with neurons and the surrounding blood vessels. Oligodendrocytes wrap myelin around axons to speed up neuronal transmission. Astrocytes extend processes that ensheath blood vessels and synapses. Microglia keeps the brain under surveillance for damage or infection (from Allen and Barres, 2009).

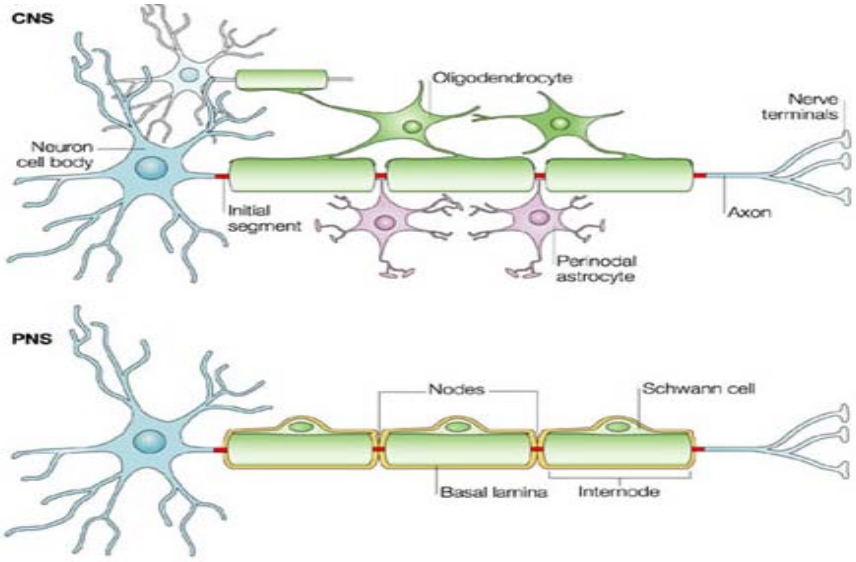


Figure 2 - *Structure of myelinated axons*. Myelinating glial cells, oligodendrocytes in the central nervous system (CNS) or Schwann cells in the peripheral nervous system (PNS), form the myelin sheath by enwrapping their membrane several times around the axon. Myelin covers the axon at intervals (internodes), leaving bare gaps — the nodes of Ranvier. Oligodendrocytes can myelinate different axons and several internodes per axon, whereas Schwann cells myelinate a single internode in a single axon (adapted from Poliak and Peles, 2003).

1.1.2 How Neurons Communicate

Communication between neurons is dependent on the properties of neuronal membranes. In a normal situation, substances will move from areas of high concentration to areas of low concentration (osmosis law), until they reach an equilibrium. However, in neuronal membranes, differences between intra and extracellular environments, prevents molecules to "walk" freely from one side to the other (Anthea et al., 1997). The diffusion of these molecules is due to their attachment to proteins that form ions channels through which some ions, such as sodium (Na+), chloride (Cl-), potassium (K+) and calcium (Ca2+), can diffuse. These transmembrane proteins or pumps transport ions bidirectionnaly. For example, the sodium-potassium pump uses transporter molecule that forces the Na^+ to leave the cell and K^+ to entry the cell. Due to these pumps, it is possible to say that neurons have at least two moments: a moment where the neuron is "resting", where there is a greater concentration of K^+ inside the cell than outside, and a greater concentration of Na^+, Cl^- and Ca^{2+} outside the cell than inside, and a second one, where any changing in the permeability of the membrane will cause an influx or an efflux of these

ions, until the system establish a balance between the inside and the outside of the cell. Due to the electrical charge of the ions, this concentration gradient will create an electrical potential (about -70 millivolt) between the two sides of the cell. The movement of ions across the cell membrane is controlled by both chemical and electrical gradients. To carry out any cognitive or motor task, whether memory formation or the execution of a movement, neurons will evaluate the inputs arriving in the form of ever-changing combinations of synaptic potentials, to determine if and when an action potential should be initiated. Neurons are polarized cells characterized by their membrane domains, including a single extensive axon and multiple dendritic processes which contain thousands of individual synapses (Figure 3a). Synapses are specialized junctional structures through which neurons communicate. According to the direction of synaptic transmission, neurons can be classified as “pre-” or “post-” synaptic neurons (Figure 3b). Synapses are composed of a pre-synaptic terminal, a synaptic cleft and a postsynaptic specialization (Kandel et al., 1991).

The process of synaptic transmission involves neurotransmitter releasing from the presynaptic nerve terminal, into the synaptic cleft that interact with postsynaptic membrane that gate ion channels (Figure 4). For example, the release of glutamate will open postsynaptic Na^+ channels, and thus the influx of Na^+ will decrease the electrical potential at the channels location. This local depolarization is referred to as an excitatory

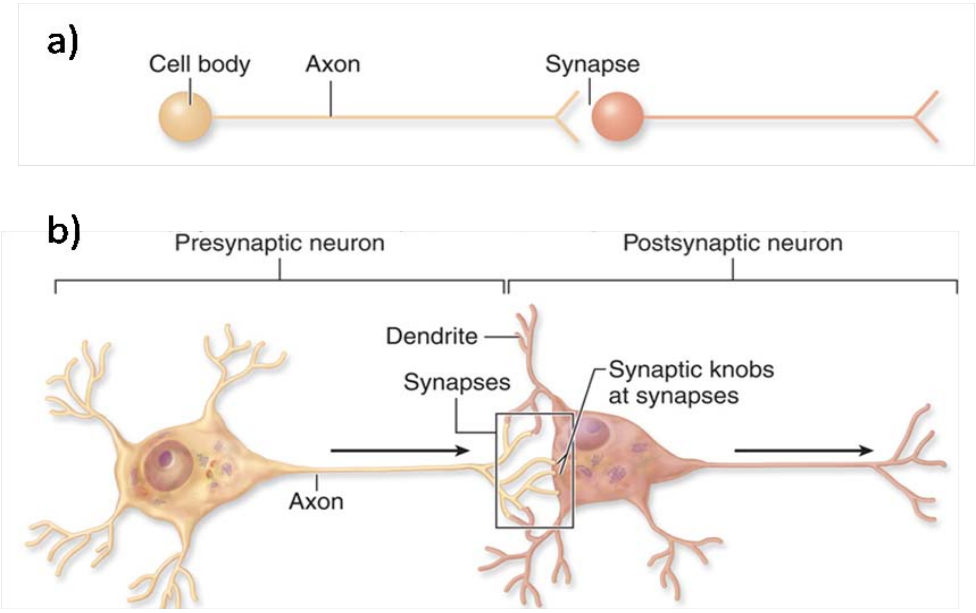


Figure 3 – *The synapse*. a) Simplified representation of a synapse; b) The prefixes "pre-" and "post-" reflect the direction of synaptic transmission: presynaptic is the transmitting side (synaptic knob) and postsynaptic is the receiving side (dendrite, soma, or effector).

postsynaptic potential (EPSP). On the other hand, neurotransmitters as GABA (gamma aminobutyric acid) exhibit inhibitory effects, as they interact with receptors to open Cl^- and K^+ channels. The influx of Cl^- or exflux of K^+ results in an increase in the resting potential at the channels location. This local hyperpolarization is referred to as an inhibitory postsynaptic potential (IPSP) (Katz, 1969). As illustrated in figure 4, the release of the neurotransmitter from the presynaptic terminal involves (1) depolarization of the terminal and (2) the presence of ions in the extracellular fluid.

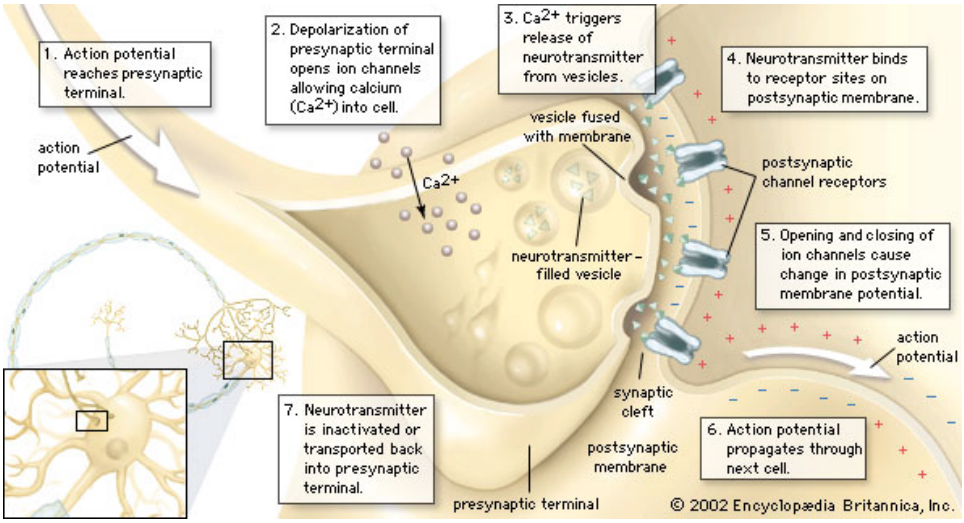


Figure 4 – Chemical transmission of a nerve impulse at the synapse (from 2002 Encyclopædia Britannica, Inc).

1.1.3 Basic Mechanisms of Axonal Transport

The mammalian nervous system includes billions of neurons, which are interconnected in several neural circuits (Horowitz el al., 1999). Neuronal function and survival involves cytoskeletal elements and constant transport of proteins and organelles to and from the cell body. The proteins used include kinesin, which is specific for anterograde transport, and dynein for retrograde transport (Ström et al., 2008).

Neuronal cytoskeleton comprises microtubules, actin and intermediate filaments (Figure 5). Microtubules and actin filaments provide the neuron with structural support, but also give conduits for intracellular transport (Ström et al., 2008; Chevalier-Larsen and Holzbaur, 2006). Microtubules provide long-range pathways for fast-anterograde movement (from the cell body to the axon terminals) of kinesin motor proteins and the

retrograde movement (toward cell body) of the dynein motor complex, whereas actin filaments are used by myosin motor proteins for short-range, dispersive distribution of vesicles, and\or organelles to the cell periphery. Roughly speaking, cargos are transported along microtubules and then transferred to the actin cytoskeleton for the final part of their journey (Akhmanova and Hoogenraad, 2005; Lansbergen and Akhmanova, 2006).

As discussed above, anterograde transport is mediated by kinesin-family proteins and is used in the translocation of membranous organelles (e.g., mitochondria) and vesicles as well as of macromolecules, such as actin, myosin, and clatrin, and some of the enzymes necessary for neurotransmitter synthesis at the axon terminals. In turn, retrograde transport is mediated by cytoplasmic dyneins and include transport of protein building blocks of neurofilaments, subunits of microtubules, soluble enzymes and materials taken up by endocytosis (e.g., viruses and toxins) (Oztas, 2003).

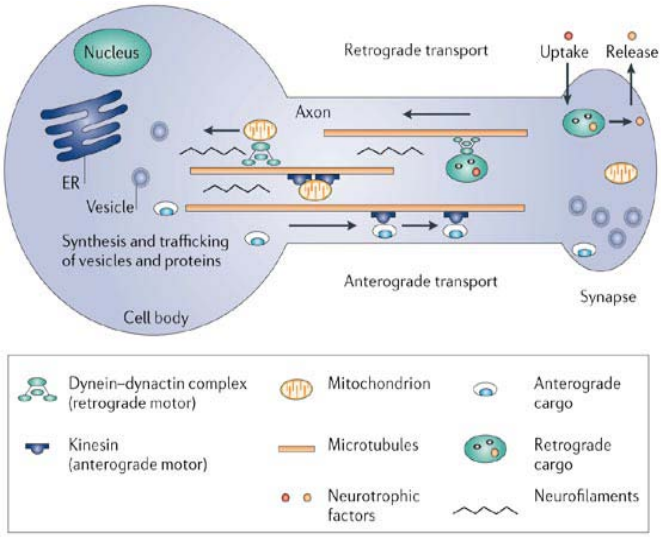


Figure 5 – *Axonal transport on microtubules*. The motors for anterograde and retrograde fast axonal transport are the kinesins and dynactin complex proteins, respectively; microtubules provide the tracks for these motors. Vesicles for transport are sorted and loaded onto transport motors both in the cell body and the distal nerve terminal. The former are transported not only into the axon but also into dendrites. Those in the distal nerve terminal permit uptake and axosomatic movement of substances such as trophic proteins. Mutations in dynactin (humans), dynein (mice) and three different forms of kinesin all provoke motor neuron degeneration (from Pasinelli and Brown, 2006).

The rate of transport is specific for each class of substances and also independent of electrical activity within an axon (Ochs, 1972). Fast axonal transport occurs in both anterograde and retrograde ways at a rate of 0.5–10 μm/sec and includes the transport of membrane-bound organelles, mitochondria, neurotransmitters, channel proteins, multivesicular bodies and endosomes (Shah et al., 2002). Slow axonal transport occurs only in the anterograde direction at a rate of 0.01–0.001 μm/sec, and conveys cytoskeletal components, such as neurofilaments, tubulin, and actin, as well as proteins such as clathrin and cytosolic enzymes (Heidemann et al., 1981; Shah et al., 2002).

1.3.4 A Brief Approach to Tract-tracing Neuronal Circuits

The discovery of the axonal transport triggered years of study into the structural basis behind this mechanism. Neuroanatomical tract-tracing methods are considered one of the best approaches to study connections between neurons located in different areas of the nervous system and to obtain data on the processing of information within a particular area (Merighi and Carmignoto, 2002). These anatomical connections can be determined by using axonal tracers that rely on intracytoplasmic movement along an axon, by retrograde transport towards the neuronal cell body and dendritic tree, or by anterograde transport towards a synapse (Köbbert et al., 2000; Lanciego and Wouterlood, 2000; Reiner et al., 2000). Thus, retrograde axonal transport allows identification of the cells of origin of afferent nerve fibers to a specific target zone, whereas anterograde axonal transport show us the projection targets of groups of cells to be charted within the CNS (Köbbert et al., 2000). There is a multitude of tracers available for the study of neuronal connections: a) horseradish peroxidase (HRP) or cholera toxin subunit B (CTb) (retrograde tracers) and low or high molecular weight dextran amines (anterograde tracers); (b) micelles and/or membrane vesicles containing lipophilic dyes; and (c) fluorescein-labeled microparticles (for review see for review see Lanciego et al., 1999; Vercelli et al., 1999; Köbbert et al., 2000).

Comparing to HRP that is passively taken up by neurons, CTb binds specifically to surface receptors of neurons (GM1-ganglioside receptor) and is actively taken up and transported by the axons, which may explain the high sensitivity of CTb as a tracer (Luppi et al., 1987; George-Chandy et al., 2001). However, CTb receptors are found to be roughly distributed either in neurons, or across cell types and also in species that may influence the labelling of the neurons according to the species on study or even in the different pathways in the same animal (Sabin, 1938). Since its discovery, in 1977, CTb has been strongly chosen for retrograde studies. As a tracer, CTb produces intense retrograde labelling from small injection sites (Dederen et al., 1994; Datiche et al., 1995; Angelucci et al., 1996; Cobos et al., 2003). CTb can also be transported anterogradely, and thus, with a single injection it is possible to study the efferent and afferent inputs of a certain area in the CNS (Chen and Aston-Jones, 1995).

Biotinylated dextran amines (BDAs) are one of the tracers mostly used in anterograde tract-tracing studies of the nervous system. BDAs are known for their

versatility and sensitivity, and depending on their molecular weight, they can be used for both anterograde and retrograde studies (Fritzsch, 1993; Kaneko et al., 1996; Medina et al., 1997a). High-molecular-weight biotinylated dextran amines (BDAs; 10 kDa) provides high-quality of labeling of axons and terminals, whereas low-molecular-weight BDAs (3 kDa) provides a detailed Golgi-like retrograde labeling of neurons. The stability of the molecular structure of BDA makes them ideals for long-term storage and examination, and their visualizations can be done by simple histochemical methods. Moreover, due to its flexibility with fixatives, BDA can be visualized at light or electronic microscopic level (for review see Reiner et al., 2000).

As CTB, BDA can be delivered into the nervous system iontophoretically or pressure-injected (Reiner et al., 2000). After injection into the CNS, BDA yields extensive and detailed anterograde labeling of axons and terminals (Veenman et al., 1992, 1995; Brandt and Apkarian, 1992; Rajakumar et al., 1993). These tracers can CTb toxin B fragment, fluorescents dextran amines or intracellular labeling (review see Reiner et al., 2000).

For anatomical tracing, cells have to be “alive”, i.e. they cannot be used in fixed tissues and for long-distance tracing in juveniles and adults, or require the presence of active transport mechanisms instead of simple passive diffusion (Lanciego and Wouterlood, 2000; Reiner et al., 2000). Using different techniques of visualization, neuronal tracing not only provide information on the morphology or afferent and efferent connectivity of neurons, but may also show the synaptic contact of neurons. However, there are some problems associated to the conventional tracing methods: 1) because the tracers must be delivered to neurons by microinjection or local application, it is difficult or impossible to selectively label small populations of neurons of a given phenotype, and 2) axons passing through the application region can be damage and become labeled, leading to a misinterpretation of results (for review see Lanciego et al., 1999; Vercelli et al., 1999; Köbbert et al., 2000). Recently, it was demonstrated that it is possible to use proteins as transneuronal tracers when its expression is genetically targeted to a subset of neurons, and thus avoid these problems (Horowitz et al., 1999).

1.2 – Pain Control

1.2.1 Pain Definition

For better or worse, we all feel pain (except in some pathologies). The International Association for the Study of Pain (IASP) define pain as a sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries [International Association for the Study of Pain (IASP) - 1994 definition, reviewed in 2008]. Painful experience exists so the body can recognize that something is threatening it and leads to behavior that will remove the organism from the source of potential injury (Landrieu et al., 1990). Pain is a key process for our nervous system to learn from and react to the environment (King et al., 1997). Certain tissues have specialized sensory receptors, called nociceptors that are activated by noxious stimulus to peripheral nerves. Upon activation, they transmit the message, by action potentials and neurotransmitters release, to the spinal cord dorsal horn for processing and transmission to the brain (Costigan et al., 2009). It is known that a noxious stimulus can result in a real or potential injury, without causing pain. In same cases, noxious stimulus can lead to pain sensation, characterized as nociceptive pain. However, painful experience can be spontaneous, such as the nonnociceptive pain characterized by the reduction of the receptor thresholds as a result of alterations of the central nervous system (CNS) (Casey, 2000). According to this, nociception and pain have different means; nociception refers to the neurophysiologic manifestations produced by noxious stimulus, while pain involves the perception of an aversive stimulus, which requires the capacity of abstraction and the elaboration of sensory impulses (Millan, 1999). According to the IASP definition, the relation between pain and degree of injury is not obligatory. Alert function is applied only to an acute manifestation, i.e., the one that follows damage to the tissue. Acute pain is delimited in time and disappears with the settle of the pathological process. On the other hand, chronic pain is characterized as persistent, and is associated with chronic pathological processes (Almeida et al., 2004).

1.2.2 Peripheral Afferent Fibers

The most important fibers for pain perception are the axons of afferent nociceptors. The afferent nociceptors (Figure 6a) consist of thermal nociceptors that are activated at

temperatures above 45ºC (C-fibers) or lower than 5ºC (A -fibers), high-threshold mechanical nociceptors that transmit information indicating injurious force on the skin (Aδ- and some Aβ-fibers) and polymodal nociceptors that are activated by thermal, mechanical and chemical stimuli (C-fibers). According to their diameter, structure and conduction velocity, C-fibers are characterized as thin (0.4-1.2 µm in diameter), unmyelinated and slowly-conducting (0.5-2.0 m sec^{-1}) fibers; Aδ-fibers as medium (2-6 µm), myelinated and of intermediate velocity (12-30 m sec^{-1}) fibers; and Aβ-fibers as large (>10 µm), myelinated and fast (30-100 m sec^{-1}) fibers (Millan, 1999). Each one of these classes encodes sensory information, however they respond differentially to noxious and innocuous stimuli, in the sense that the three fibre types transmit non-nociceptive information, but only C and Aδ fibers transmit nociceptive information in the normal tissue (nociceptors; Giordano, 2005).

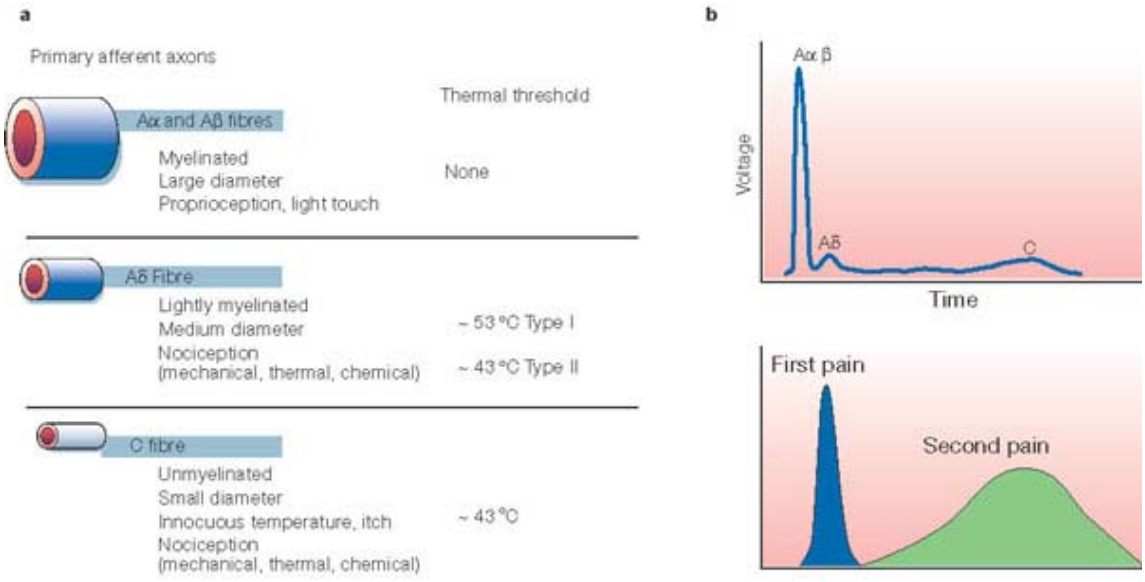


Figure 6 – *Different nociceptors detect different types of pain*. a) Peripheral nerves include small-diameter (A) and medium- to large-diameter (A,) myelinated afferent fibres, as well as small-diameter unmyelinated afferent fibres (C); b) The fact that conduction velocity is directly related to fibre diameter is highlighted in the compound action potential recording from a peripheral nerve. Most nociceptors are either A or C fibres, and their different conduction velocities (6–25 and 1.0 m s-1, respectively) account for the first (fast) and second (slow) pain responses to injury (from Julius and Basbaum, 2001).

Following a noxious stimulus, primary nociceptive afferents respond with differentiated patterns of propagation (Figure 6b). The myelinated Aδ-fibers transmit

impulses much faster than do the unmyelinated C-fibers. The Aδ-fibers transmit what is called the first pain, i.e., sharp and highly localizable. Impulses on C-fibers are responsible for what is called second pain. Second pain is slower in arriving, duller and endures after the stimulus end (Casey, 2000).

1.2.3 Nociceptors

To guard against tissue damage, it is important that the body is alerted of potentially damaging stimuli. This awareness is attained by a noxious stimulus-detecting sensory system (Costigan et al., 2009). Nociceptors are physiological receptors located all over the body - skin, internal organs, joints, muscles and tendons. When activated, either by noxious stimuli, tissue injury or acute inflammation, the propagation of nociception is initiated and afferent information is send to the dorsal horn of the spinal cord where synaptic transmission to ascending pathways is subject to modulation by descending pathways, local neuronal circuits and different kinds of neurochemicals (Figure 7) (Almeida et al., 2004). Some nociceptors are thinly myelinated (Aδ-fibers) but most are unmyelinated (C fibers), and these slowly conducting afferents represent the majority of sensory neurons in the PNS. Like all primary sensory neurons in the somatosensory system, nociceptors have their cell bodies located in the dorsal root ganglia (DRG) or trigeminal ganglia, give rise to a single axon that bifurcates into a peripheral branch that innervates peripheral target tissue, and a central axon that enters the CNS to synapse on nociceptive second order neurons. Morphologically, nociceptors are similar to other neurons; they have a peripheral terminal that transduce external stimuli and initiates action potentials, a axon that conducts action potentials, a cell body that controls the identity and integrity of the neuron and a central terminal that forms the presynaptic element of the first synapse in the sensory pathway in the CNS (Figure 7) (Woolf and Ma, 2007).

There are three major classes of nociceptors – thermal, mechanical, and polymodal – as well as a class classified as silent nociceptors. Aδ mechanical nociceptors, respond to noxious mechanical stimuli that damage or threaten to damage tissue. C-polymodal nociceptors, react to noxious mechanical, noxious thermal (>44°C) and noxious chemical stimuli. Silent (or sleeping) nociceptors, which do not respond to acute noxious stimulation of uninjured tissue, become active after tissue injury. The information from nociceptors is conveyed by sensory axons, whose cell bodies are in the dorsal root ganglion, to the spinal

cord where they synapse onto second-order spinal cord neurons, which transmit the information to supraspinal sites (e.g., the thalamus in the brain) (Willis and Westlund, 1997).

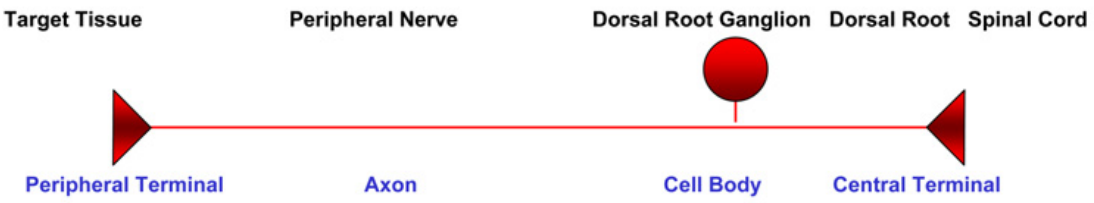


Figure 7 – *The nociceptor*. The operational components of the nociceptor include a peripheral terminal that innervates target tissue and transduces noxious stimuli, an axon that conducts action potentials from the periphery to the central nervous system, a cell body in the dorsal root ganglion, and a central terminal where information is transferred to second order neurons at central synapses (from Woolf, and Ma, 2007).

1.2.4 From Periphery to Thalamus

Pain can be understood as a complex entity that does much more than simply activate a "pain center", resulting from complex and interactive series of mechanisms integrated at all levels of the neuroaxis, from the periphery, via the spinal dorsal horn to higher cerebral structures (Talbot et al., 1991; Casey et al., 1994; Derbyshire et al., 1997; Millan, 1999).

The dorsal horn of the spinal cord is the location of the first synapse in pain pathways, and as such, offers a powerful target for regulation of nociceptive transmission (Heinricher et al., 2008). Primary afferent fibers form synapses with dorsal horn sensory neurons, which send ascending projecting fibers and make synapses with neurons located at supraspinal sites, such as the thalamic nuclei (Zhuo, 2007). Nociceptive information ascends from the spinal cord to the thalamus in the contralateral spinothalamic tract (STT), to the medulla and brainstem via the spinoreticular and spinomesencephalic tracts, to the hypothalamus via the spinohypothalamic tract, to the supraspinal autonomic control centers via the spinohypothalamic tract and to the nuclei in the midbrain, ventroposterior lateral and posteriomedial nuclei of the thalamus in the cervicothalamic tract (Millan, 1999; Willis and Westlund, 1997; Craig, 2003; Pralong et al., 2004).

1.2.5 Brainstem Control of Spinal Nociceptive Processing

The descending pain modulatory system, also known as endogenous pain control system, is a well-characterized anatomical network that enables us to regulate nociceptive processing in differents situations to produce either facilitation (pro-nociception) or inhibition (antinociception) (Hagbarth and Kerr, 1954; Fields and Basbaum, 1999; Treed et al., 1999; Heinricher et al., 2009). This modulatory system may facilitate or inhibit nociceptive input by three major mechanisms: 1) the modification of synaptic strength in the spinal dorsal horn may increase or decrease transmission of nociceptive signals to the brain; 2) local dorsal horn interneurons provide both feed-forward and feed-back modulation to spinothalamic and spinobulbar projection neurons; and 3) descending systems initiating in the brainstem exert top-down modulation of nociceptive input at the spinal level (Seifert et al., 2009).

In 1906 it was demonstrated, for the first time, that brain can modulate in a ‘‘top-down’’ way spinal cord excitability via a tonically active influence, most of the time inhibitory in function. This idea came from Sherrington work that showed that nociceptive reflexes were improved after transaction of the spinal cord (Sherrington, 1906). Later on, data from 1969 came to emphasized the relevance of this experience by showing that focal electrical stimulation in the rat midbrain periaqueductal gray (PAG) produced analgesia strong enough to allow surgery without anesthetics or analgesics (Reynolds, 1969). Electrophysiological, anatomical, and pharmacological studies have shown that these descending influences on spinal nociceptive processing were modulated at the rostral ventromedial medulla (RVM), which includes the medial nucleus raphe magnus (Porreca et al, 2002; Gebhart, 2004). The RVM receives inputs from the PAG and, in turns, projects to the dorsal horn, primarily to the superficial layers, where it can influence spinal nociceptive transmission. RVM cells have two major types of neurons that may explain the role of the RVM in pain modulation: ON-cells, which facilitate nociception via descending axons projecting to the spinal cord (pro-nociception), and OFF-cells, which inhibit nociceptive information directly at the level of the spinal cord (antinociception) (Fields et al., 1983; Fields and Heinricher, 1985).

The antinociceptive nature of brainstem areas, such as the PAG (Bodnar, 2000), the RVM (Mason, 2001), the locus coeruleus (LC; Jones, 1991), the lateral portion of the caudal ventrolateral medulla (VLMlat; Tavares and Lima, 2002), the dorsal reticular nucleus (DRt; Bouhassira et al., 1992; Almeida et al., 1996; 1999), and the nucleus tractus solitarius (NTS; Randich et al., 1988) is well established in the literature. The inhibitory antinociceptive nature of the system was latter questioned by the observation of pronociceptive effects from areas classically considered as antinociceptive, as the RVM (Porreca et al., 2002), the NTS (Wiertelak et al., 1997) and the DRt (Almeida et al., 1996; Almeida et al., 1999; Dugast et al., 2003), which play an additional profound nociceptive facilitating effect upon acute, inflammatory and chronic pain (Sotgiu et al., 2008). The idea of a primary pronociceptive centre in the endogenous pain control system leaded to a new concept of pain modulation as a dynamic and flexible process, resulting from balance between excitatory and inhibitory actions as the way of adapting to the various unsteady pain determinants (Lima and Almeida, 2002). In conclusion, pain control results from the balance between inhibiting and facilitating outflows from the brainstem upon spinal nociceptive transmissions and a disruption of this balance may constitute the basis for

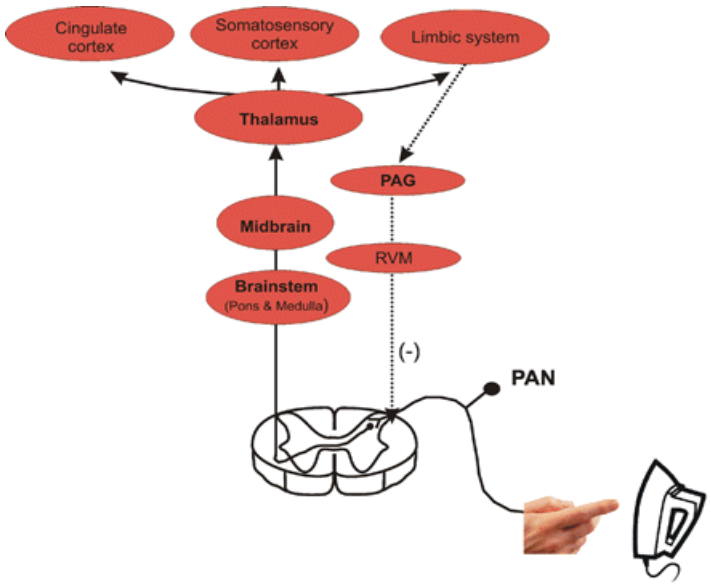


Figure 8 – *The neural pathway of nociception from primary afferent neurons (PANs) to the superficial lamina in the dorsal horn of the spinal cord*. Second-order neurons in the dorsal horn convey the noxious signal to the brainstem, midbrain, and thalamus. Finally, third-order neurons relay the electrical signal to the somatosensory/cingulate cortex and limbic system. Descending modulatory influences arrive in the spinal cord dorsal horn (dashed lines) and are derived from the midbrain periaqueductal gray (PAG), the locus ceruleus, and the rostral ventromedial medulla (RVM) (from White et al., 2007).

chronification of pain (Urban and Gebhart, 1999; Porreca et al., 2002; Vanegas and Schaible, 2004; Heinricher et al., 2009).

1.3 – The Brainstem Reticular Formation and Pain

1.3.1 The Brainstem Reticular Formation

The reticular formation (RF) is the name given to the collection of small nuclei and fiber tracts that run through the core of the brainstem. The RF extends from the caudal medulla, where it is continuous with the spinal cord reticular formation, to the mesencephalon (Figure 9). The different RF areas receive afferents from most of the sensory systems and projects for almost all parts of the nervous system (Hendelman, 2000). Several studies demonstrated that some subgroups of reticular neurons that receive inputs from peripheral receptors, including skin, muscle, bone and joint receptors, integrate them and link to the vestibular and cerebellar circuits that will determine posture and movement. This information is also integrated by higher brain structures in the visual, somatosensory, and motor systems to develop the complex motor patterns of adaptive behaviour (Squire, 2003).

The heterogenic morphology of the RF may explain the multifaceted role in the CNS; it exerts important functions on the sleep/wake cycle, regulation of visceral activity, control of movement, behaviour, alertness and modulation of pain (Rhoades and Bell, 2008). According to the function performed, it is possible to delimit different subgroups within the RF:

- Cardiac and respiratory “centers”: subsets of neurons within the medullary reticular formation responsible to control the vital functions of heart rate and respiration;
- Motor areas: the motricity is controlled by both pontine and medullary nuclei of the RF via the cortico-reticulo-spinal system;
- Ascending projecting system: fibers from the RF ascend to the thalamus and project to differents nonspecific thalamic nuclei. Here, fibers ascend diffusely to the cerebral cortex. This system is related with consciousness and has been termed reticular activating system (ARAS);

- Pre-cerebellar nuclei: several nuclei in the brainstem placed within the boundaries of the RF that project to the cerebellum.

A different way to describe this area is according to the spatial positioning of the neurons. Topographically, neurons can be arranged in three longitudinal sets, each of them with different cytoarchitecture, connections and physiological functions. The lateral group is formed by small neurons that receives inputs to the RF, including those from the

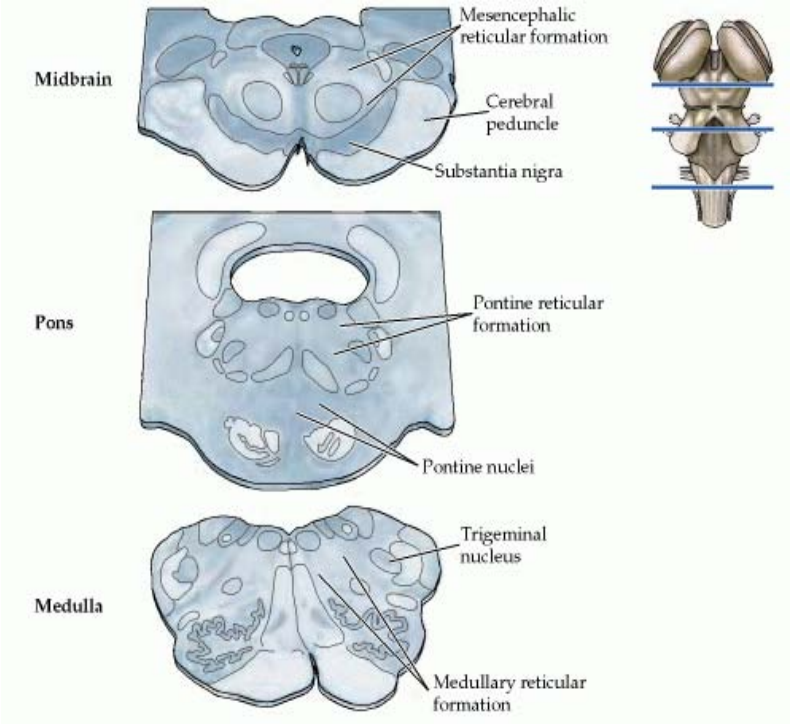


Figure 9 – *The brainstem reticular formation*. The location of the reticular formation in relation to some other major landmarks at different levels of the brainstem. Neurons in the reticular formation are scattered among the axon bundles that course through the medial portion of the midbrain, pons, and medulla (from Purves et al., 2007).

anterolateral (pain and temperature) and trigeminal systems, and the auditory and visual input (Kiernan J., 2008). The central group consists of big neurons, and gives rise to long ascending and descending fibers pathways, some of them projecting both rostrally into the thalamus and caudally to the sacral levels of the spinal cord, thus influencing the axial and proximal limb muscles. Within this group are the nucleus gigantocellularis of the medulla and the pontine reticular nuclei, positioned at caudal (lower) and oral (upper) parts, forming the two reticulo-spinal tracts. Several staining studies have shown that the medial RF contains medium-to-giant-bodied neurons characterized by far-reaching bifurcating axons running rostro-caudally, with long branches contacting either the forebrain or spinal

cord (Scheibel and Scheibel, 1967; Newman, 1965). These cells are referred as projection neurons based on the idea that all of them project outside the medial RF. Electrophysiological studies have demonstrated that synaptic connections between these neurons, formed by terminals of the axons collaterals, are enough to induce excitatory post-synaptic potentials (Ito and McCarley, 1987), suggesting a anatomically and functionally connection between these projection neurons (Humphries et al., 2006). Some evidences suggests a second cell-type in the medial RF, referred as interneurons, however not conclusive (Ito and McCarley, 1987). Finally, the midline region is occupied by a group of neurons, known as raphe nuclei. The nucleus raphe magnus is one of the nucleus of this group, which plays an important role in the descending pain modulatory system (Hendelman, 2000).

1.3.2 Neurotransmitters in the Reticular Formation

Chemical neurotransmitters have been identified and localized in groups of cells within the RF, acting in cortical activation and behavioural arousal. Norepinephrine (NE) is contained in neurons of the pons and medulla (A1-A7 cells groups). The A5 and A7 cells groups (in rostral part) projects caudally to the brainstem and spinal cord, whereas the A1-A3 cell groups (in caudal part) projects rostrally to the brainstem, hypothalamus and basal forebrain. Together, they form the lateral tegmental area, apparently involved in hypothalamic regulation and motor control (Moore and Card, 1984). Serotonin or 5-HT is contained in neurons located in the midbrain nuclei, dorsal raphe nuclei, and the median raphe nuclei (B8 and B9). These neurons project rostrally, innervating nearly the entire forebrain, thus suggesting a role in regulation of behaviour state. The acetylcholine (ACh) is known as the neurotransmitter of motor neurons, and apparently is associated to nonmotor brain areas. Cholinergic neurons may be found either in the pontine nuclei (the laterodorsal tegmental nucleus and the pedunculopontine nucleus), projecting to the brainstem RF, hypothalamus, thalamus and basal forebrain, or in the medial septum (nucleus of the diagonal band and the substantia innominata-nucleus basalis complex), which projects to the limbic forebrain, including the hippocampus, and to the neocortex (Squire, 2003). In summary, these neurotransmitters are produced by modulatory neurons, whereas neurons involved in sensorimotor integration produce either the excitatory transmitter glutamate or the inhibitory transmitter GABA (Squire, 2003).

1.3.3 The Ascending Reticular Activating Systems Mediates Consciousness and Arousal

The RF receives sensory information from many systems of the body and it is broadly interconnected with the cerebellum and the limbic system (Gilman et al., 2003). The limbic system consists of a group of deep brain structures, including the hippocampus, amygdala, gyrus fornicatus and connecting structures, which support a variety of functions such as emotion, behaviour, long term memory, and olfaction. Nearly all the neural fibers carrying information into and out of the RF have a crucial role in initiation and control of sleep-wake cycle (Siegel, 2002).

Individual reticular neurons communicate with higher brain areas such as hypothalamus, thalamus, cerebellum and spinal cord, making reticular neurons ideal for leading the arousal of the brain as a whole. For example, some reticular neurons, if not inhibited by other brain areas, are sending continuously impulses (via thalamic relays) to the cerebral cortex, keeping it alert and conscious and improving its excitability. This function of the RF is called the reticular activating system (RAS) also known as "activating system" and its activity is crucial for maintaining the state of consciousness and sleepiness (Marieb and Hoehn, 2007). The interest in the sleep-wakefulness cycle begins in 1949, when Giuseppe Moruzzi and Horace Magoun discovered that by stimulating the reticular formation, they could awaken animals from normal sleep (Butkov and Lee-Chiong, 2007). On the same year, Donald Lindsley authored a paper showing that the loss of arousal was related to the rostral section of the RF in the mesencephalon (Siegel, 2002). However, in 1950, a new study on cats, has shown that these animals were capable to be awakened from coma with auditory and tactile stimuli, despite the destruction of large neuronal fiber tracts that where projecting from the RF to thalamus and then to the cortex (Siegel, 2002).

The RAS consist of specific thalamic nuclei, parts of the hypothalamus, the ventral tegmental area, the parabrachial nuclei, the periaqueductal gray, the nucleus locus coeruleus, the raphe nuclei, and the RF itself, most of them involved in arousal and cortical tone modulation (Solms and Turnbull, 2002). Impulses coming from ascending sensory tracts synapse with RAS neurons, maintaining them active and improving their arousing effect on the cerebrum. These pathways involve three to four synapses: a peripheral receptor responds to a specific sensory stimulus, as touch, hearting or vision, and transmits the information to cells of the RF that projects to the intralaminar nuclei of thalamus,

which innervate large areas of the cerebral cortex and limbic system (Rhoades and Bell, 2008). This system is inhibited by sleep centers located in the hypothalamus and other neural regions, and is depressed by alcohol and drugs, including tranquilizers. RAS stimulation makes the cortex more alert and aware. However, any injury to the brain, mainly in the brainstem, like head injuries, oxygen deprivation of the brain, drugs, and electrolyte changes can influence these centers, resulting in permanent unconsciousness or even coma (Marieb and Hoehn, 2007).

RAS can be divided into two components: the first one, ventral, lower and arousal circuit, running parallel to the reticular-thalamic-cortical circuit, and the second one, more rostral, is in the basal forebrain, making part of the ventral arousal circuit (for more detail see book Marieb and Hoehn, 2007). Besides RAS, there is also a descending element of arousal, which function as a physiological activator of the body, and supporting increase in activity that normally occurs with waking (Siegel, 2002). These two systems functioning together form a positive loop that, if uncontrolled, can result in a severe arousal state (Butkov and Lee-Chiong, 2007). In summary, the reticular activating system is responsible to transmit sensory input. Nerve impulses, as light and noise, project from the cerebral cortex and course via thalamus, stimulating the reticular activating system, resulting in arousal and awaken. The sleep-wakefulness cycle results from a feedback system of communication between the reticular activating system and the cerebral cortex (Butkov and Lee-Chiong, 2007).

Additionally, some nuclei of the RF are motor nuclei, which project to motor neurons in the spinal cord through the reticulospinal tracts. Some nuclei assist in the control of skeletal muscles during coarse limb movement, and other, as vasomotor, cardiac, and respiratory centers of the medulla, function as autonomic centers regulating visceral motor functions (Marieb and Hoehn, 2007).

1.3.4 The Reticular Formation and Nociception

It is know that nuclei from the reticular formation play an important role in the processing of nociceptive information (Bowsher, 1976). This idea is based on anatomic studies performed in various species (Villanueva et al., 1988), including man (Bowsher, 1957; Bowsher, 1962), where most of spinal afferents that travel through the anterolateral

quadrant terminate within the brainstem reticular formation. In addition, several areas throughout the brainstem reticular formation contain neurons responsive to noxious stimuli (Villanueva et al., 1988). For example, neurons recorded within the nucleus gigantocellularis are activated by noxious mechanical, electrical, or chemical stimulation of their peripheral receptive fields (Casey, 1969; Goldman et al., 1972; Guilbaud et al., 1973; Gokin et al., 1977; Leblanc and Gatipon, 1974; Pearl and Anderson 1978). On the other hand, focal electrical stimulation of this area has been shown to elicit escape behaviour (Casey, 1971). Other authors have also reported the existence of neurons responding exclusively to noxious mechanical, thermal, or electrical stimulation in more caudal areas, such as the caudal bulbar reticular formation (Benton, 1968; Benjamin, 1970; Rose, 1975; Mayer and Hill, 1978; Blair, 1985); others have shown that neurons in this region can also respond to high threshold visceral stimulation (Gokin et al., 1977) and noxious cardiac stimulation (Blair, 1985).

RF structures have a well-characterized role in descending modulation of pain. For instance, the RVM is involved in the development and maintenance of central sensitisation and secondary hyperalgesia in animals (Urban and Gebhart, 1999). The PAG and the nucleus cuneiformis (NCF) are the main sources of input to the RVM (Basbaum and Fields, 1984; Behbehani and Zemlan, 1986), and are in an ideal position to modulate its output, i.e. modulate spinal nociception. These nuclei have a physiological substrate for bidirectional modulation of pain processing, due to different functionally classes of cells, which either facilitate (ON-cells) or inhibit (OFF-cells) nociception (Fields et al., 1983; Haws et al., 1989; Heinricher et al., 1987). Later on, other nuclei were shown to have anatomical inter-bulbar and bulbo-spinal connections (Lee et al., 1991; Mtui et al., 1993, Esteves et al., 1993, Tavares and Lima, 1994, reviewed by Tavares and Lima, 2002, Lima and Almeida, 2002; Almeida et al, 2006). Areas like the DRt are involved in modulatory actions that are based on a reciprocal circuitry with the spinal cord that allows a rapid adaptation to any change occurring in the system (reviewed by Tavares and Lima, 2002, Lima and Almeida, 2002, Almeida et al., 2006).

1.3.5 The ventral reticular nucleus

The ventral reticular nucleus (VRt) remains a relatively unexplored area of the caudal medullary reticular formation, contrary to its dorsal counterpart (DRt), whose

involvement in pain modulation is well established. The VRt is the caudal continuation of the nucleus reticularis gigantocellularis and continues caudally through the deep lamina of the spinal dorsal horn. It is located ventrally to the trigeminal subnucleus caudalis and the DRt. The potential involvement of VRt in nociceptive processing was initially proposed based on the fact that VRt neurons could be activated bilaterally by noxious stimulation of the face (Yokota et al., 1991). VRt neurons project mainly to the ventral horn (laminae VII-X), via the ventral funiculi and to laminae IV-V (Tavares and Lima, 1994; Villanueva et al., 1995) and receives projections from laminae V-VII (Raboisson et al., 1996).

VRt is also connected with other reticular areas, including the VLM (Cobos et al., 2003) and the DRT (Almeida et al., 2002). The participation of VRt in pain modulation has been demonstrated by electrophysiological studies. The first one reported two different groups of VRt neurons: 1) neurons exhibiting spontaneous activity which was unaffected by innocuous mechanical stimulation and either unaffected or inhibited by noxious peripheral mechanical stimulation and 2) neurons displaying regular, rhythmic activity which was synchronous with the rate of ventilation (Villanueva et al., 1988). A second study shows that electrical stimulation of the VRt induced analgesia and attenuation of the responses of spinal neurons (Aicher and Randich, 1990). A third study reported that animals with lesions in VRt seem to feel more pain in behavioral tests, which is the opposite of results from lesioning the DRt, thus excluding VRt involvement in the facilitation of nociception and suggesting its involvement in antinociception (Almeida et al., 1999).

This thesis aims to analyze the overall pattern of brain connections of the VRt, using local iontophoretic injections of the anterograde tracer biotinylated-dextran amine (BDA) or the retrograde tracer cholera toxin subunit B (CTb).

Chapter 2

EXPERIMENTAL PROCEDURES

2.1 – Ethical guidelines

Surgical procedures were performed under pentobarbital anesthesia (50 mg/kg, i.p.) on Wistar male rats (Charles River Laboratories, Barcelona, Spain) weighing 280–320 g. Animals were placed in a stereotaxic device (Stoelting, Wood Dale, IL, USA) and a craniotomy was performed. Coordinates for brain injections followed the stereotaxic parameters of (Paxinos and Watson, 1998). The experiments were in accordance with the regulation of local authorities for handling laboratory animals and the European Community Council Directive 86/609/EEC. The number of animals used and their suffering were minimized.

2.2 – Anterograde tracing experiments

Twenty one rats received iontophoretic injections (positive direct current of 3.0 µA; 5 s on/5 s off, lasting for 10 min) of 10% BDA (10,000 MW; Vector Laboratories, Burlingame, USA) in the left VRt through glass micropipettes with 15–20m diameter tips. After completion of the injection period the micropipettes were left in situ for 10–15 min before being slowly retracted to avoid tracer reflux along the pipette tract. Two to three weeks later, animals were reanesthetized with eutasil (1 mL/kg body weight) and perfused through the ascending aorta, first with 100 mL of saline phosphate buffer (PBS) 0,1 M, pH 7.2 and then with 1000 mL of 4% paraformaldehyde in PBS. The entire brain was removed, immersed in the same fixative for 4 h (RT) and then in 8% sucrose in PBS at 4 °C for 1–2 days. Coronal sections of the entire brain were serially cut on a vibratome at 50 µm and incubated with 3.3% H_2O_2 in order to inhibit endogenous peroxidase. Two in every three successive brain sections were immunoreacted with avidin–biotin complex (ABC, 1:200; Vector Laboratories) for 1 h and then BDA was revealed with 0.0125% diaminobenzidine tetrahydrochloride (DAB; Sigma Immunochemicals, St. Louis, USA) and 0.02% H_2O_2 in Tris–HCl buffer 0.05 M, pH 7.6. Half of these sections were counterstained using the formol-thionin technique (Donovick, 1974) and the remaining was left without any counterstaining. Sections with and without counterstaining were then serially placed in SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany), dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

2.3 – Retrograde Tracing Experiments

Twenty Wistar male rats were iontophoretically injected with 1% CTb (List Biological Products, Campbell, CA, USA) using the same procedures described above for BDA. One week after the injection they were reanesthetized and perfused as above. After the inhibition of endogenous peroxidase, serial brain sections were left overnight at 4 ºC in a goat antibody against CTb (List Biological Products) at 1:40,000 in 0.1 M PBS containing 0.3% Triton X-100 (PBST). After several washes in PBST sections were incubated for 1 h in PBST containing a biotinylated anti-goat antibody raised in horse (1:200; Vector Laboratories). Sections were washed again in PBST and then incubated in PBST containing ABC (1:200). This and subsequent steps were similar to those described above for BDA experiments.

2.4 – Image analysis and illustrations

All the photographic material presented in this study was obtained using a digital camera (AxioCam HRc) connected to a microscope (Axioskop 2 Plus), both from Carl Zeiss (Göttingen, Germany). Images were captured in a computer using AxioVision 3.1.2.1 software and the brightness/contrast of each image was improved using Adobe Photoshop 7.0.1. software. For illustrative proposes, the brain areas receiving efferent projections from the VRt were drawn using a sequence of selected formol–Thionin-stained coronal sections of one illustrative animal injected with BDA. A motorized microscope (Axioplan2, Carl Zeiss) connected to a digital camera (Sony 3CCd DSP, Japan) was used to capture the image of the selected brain sections. For each section, the limits of the brain nuclei and the labeled fibers were drawn under a 1.25x or 40x objective lens, respectively, using Stereo Investigator 4.34 software (MicroBrightField, Inc, Willinston, VT, USA).

The nomenclature/abbreviations used to designate brain nuclei and fiber tracts are in accordance with those used by Paxinos and Watson (1998, 2005) or result from a simplification of it.

Chapter 3

RESULTS

3.1 – Injection sites

Of the 21 animals that received iontophoretic BDA injections, 5 had injection sites that were located in the VRt. All of the BDA injection sites presented a central core of labelled neuronal somata and fibers surrounded by a peripheral region containing scattered neuronal perikarya with dendrites that extended into the core, resulting from retrograde transport. Only those injections whose central dark core and surrounding halo were located inside the VRt were considered valid for the present study. Two representative injection sites are illustrated in figure 1: animal UMHugo23 (Figure 10A), located medially to Sp5C, ventrally to IRt and dorsal medially to LRt, and UMHugo24 (Figure 10B), more centred within the nucleus. The injection centers are small and as shown in Figures 1 and 3, they are found to be located within the boundaries of the VRt as defined by Paxinos.

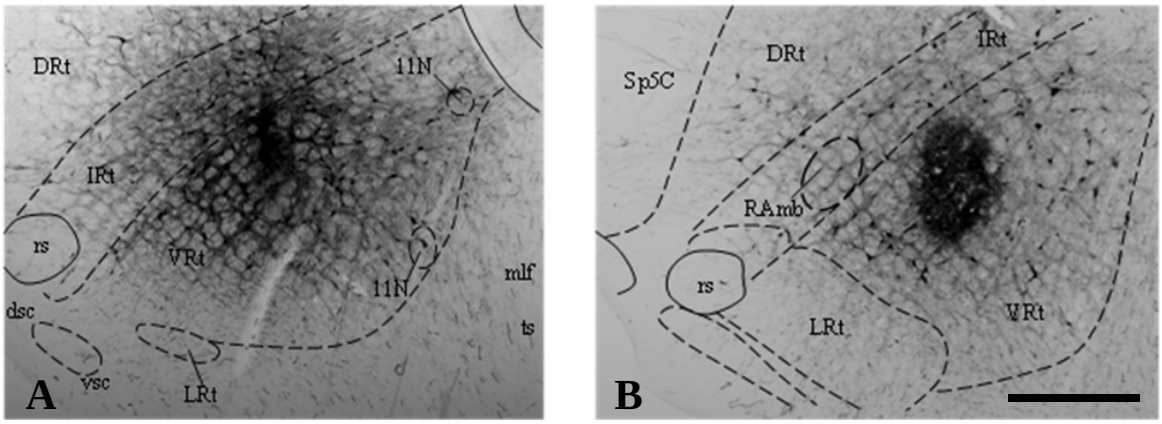


Figure 10 - Photomicrographs of representative iontophoretic BDA injections in the VRt. Scale bar = 200 μM

As to the CTb injections, from the 20 animals that were injected only 2 had injection sites located in the VRt. CTb injection sites appeared as a compact dark zone, bounded by a halo in which dark areas intermingled with lighter zones. Nearby the peripheral halo were observed a small number of retrogradely labelled cells, probably due to uptake from the more central areas. According to previous studies (Ericson and Blomqvist 1988; Lima et al., 1991), the injection sites will be delimited only by the central core and the peripheral halo. Selection of CTb injection (Figure 11) followed the same principle.

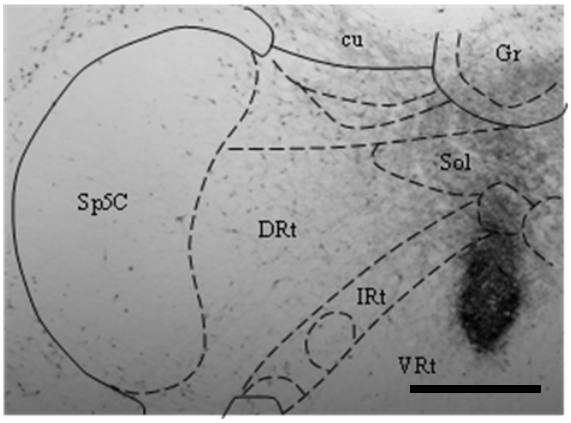


Figure 11 - Photomicrographs of a representative iontophoretic CTb injections in the VRt. Scale bar = 500 µM.

3.2 – Anterograde Tracing Experiments

BDA administration to the VRt. Following BDA injections restricted to the VRt, anterogradely labelled fibers and terminal boutons appeared along the medulla oblongata, pons, mesencephalon, and in some restricted areas of the diencephalon (Figure 12). BDA labelled fibers were found mainly ipsilaterally to the injection, with a contralateral predominance to diencephalic areas (Figure 13). A detailed analysis of the brain areas receiving projections from the VRt will be further presented.

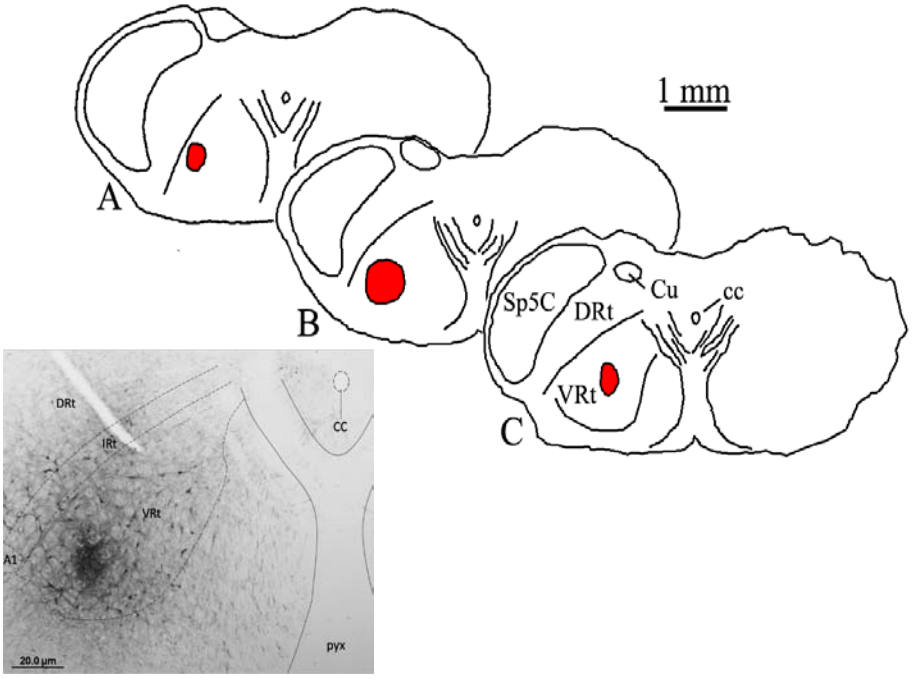


Figure 12 - Camera lucida-like drawings of a representative BDA injection along three successive rostro-caudal (A-C) levels of the VRt. Red areas represent the core of the injection. The same injection is depicted in the photomicrograph.

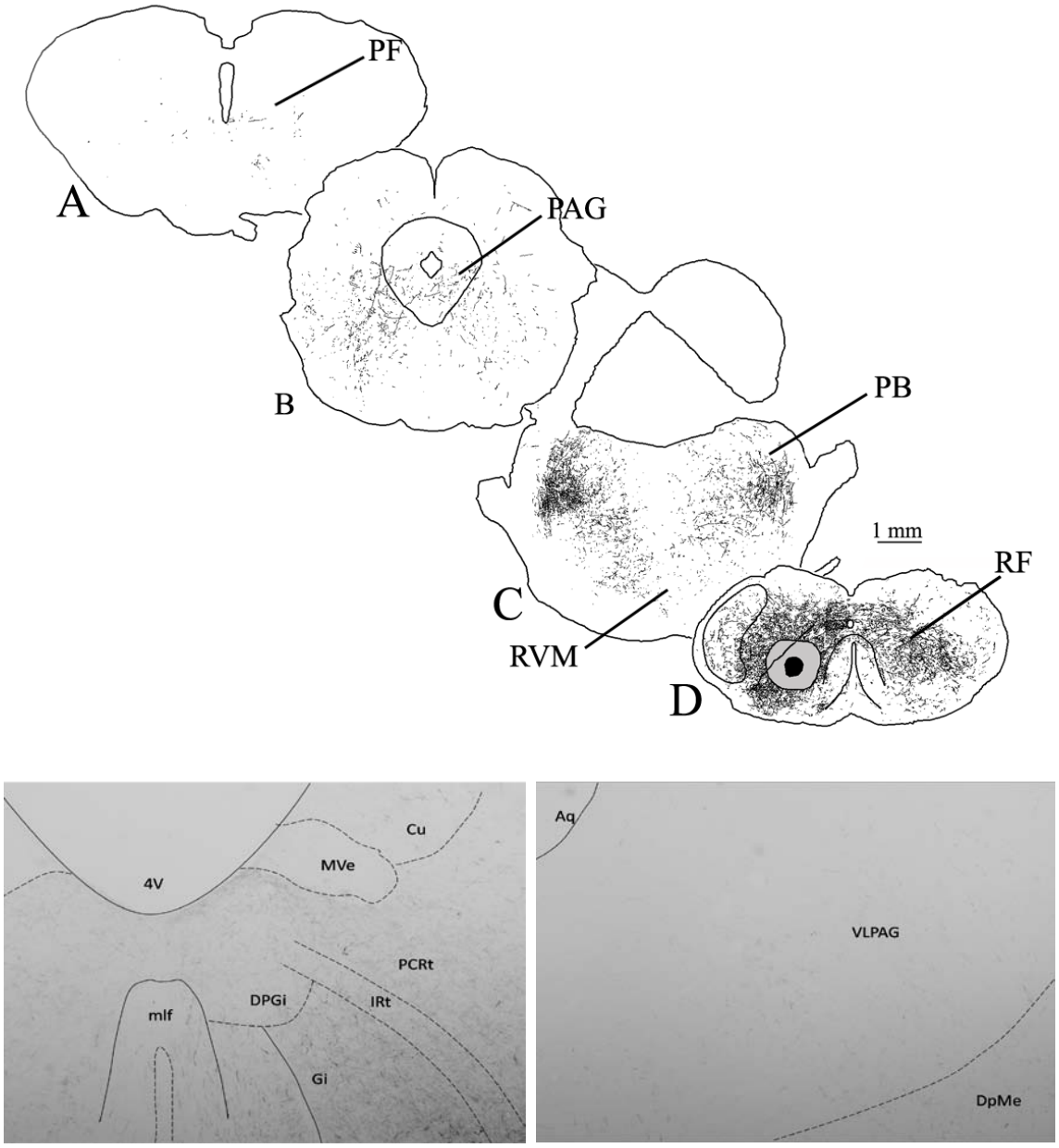
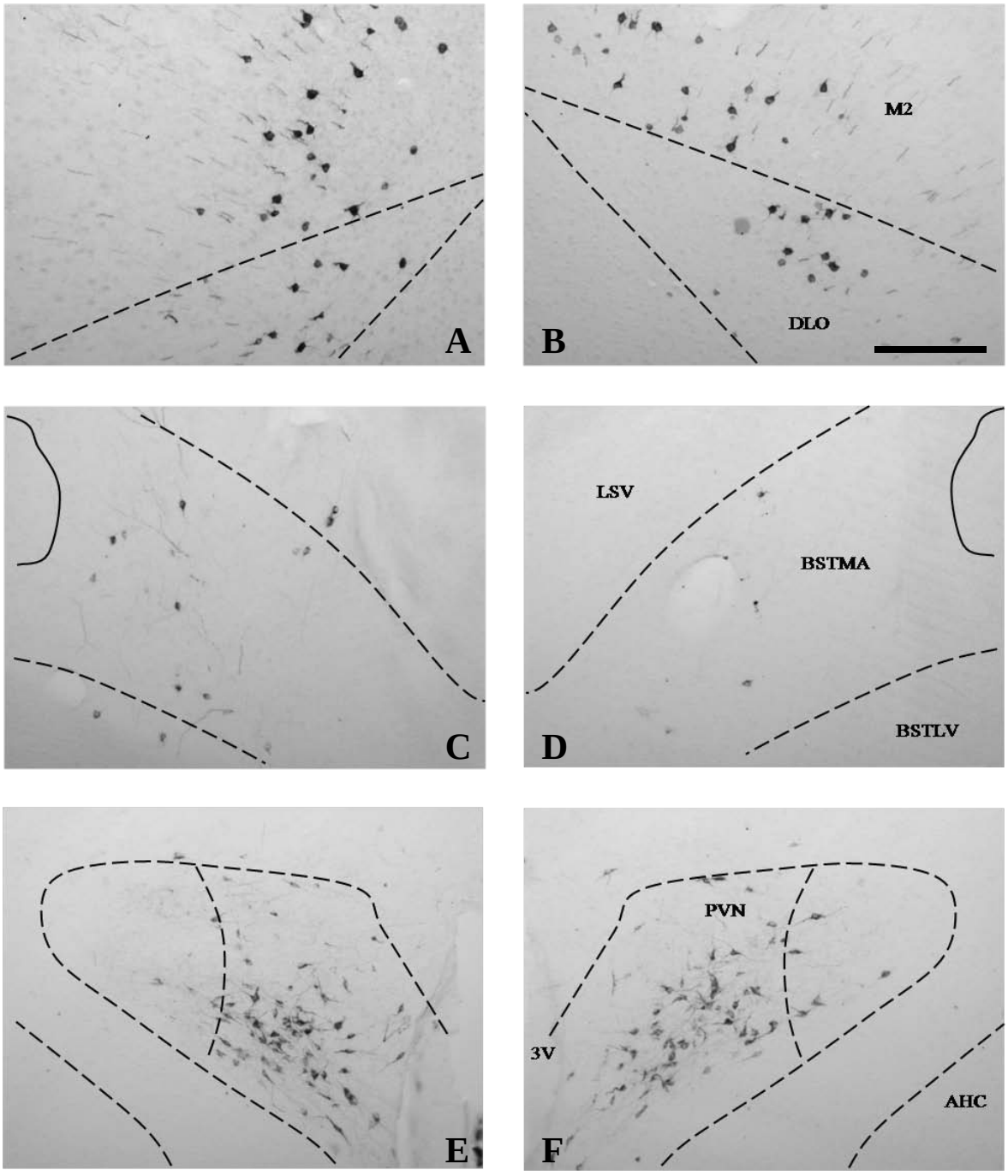


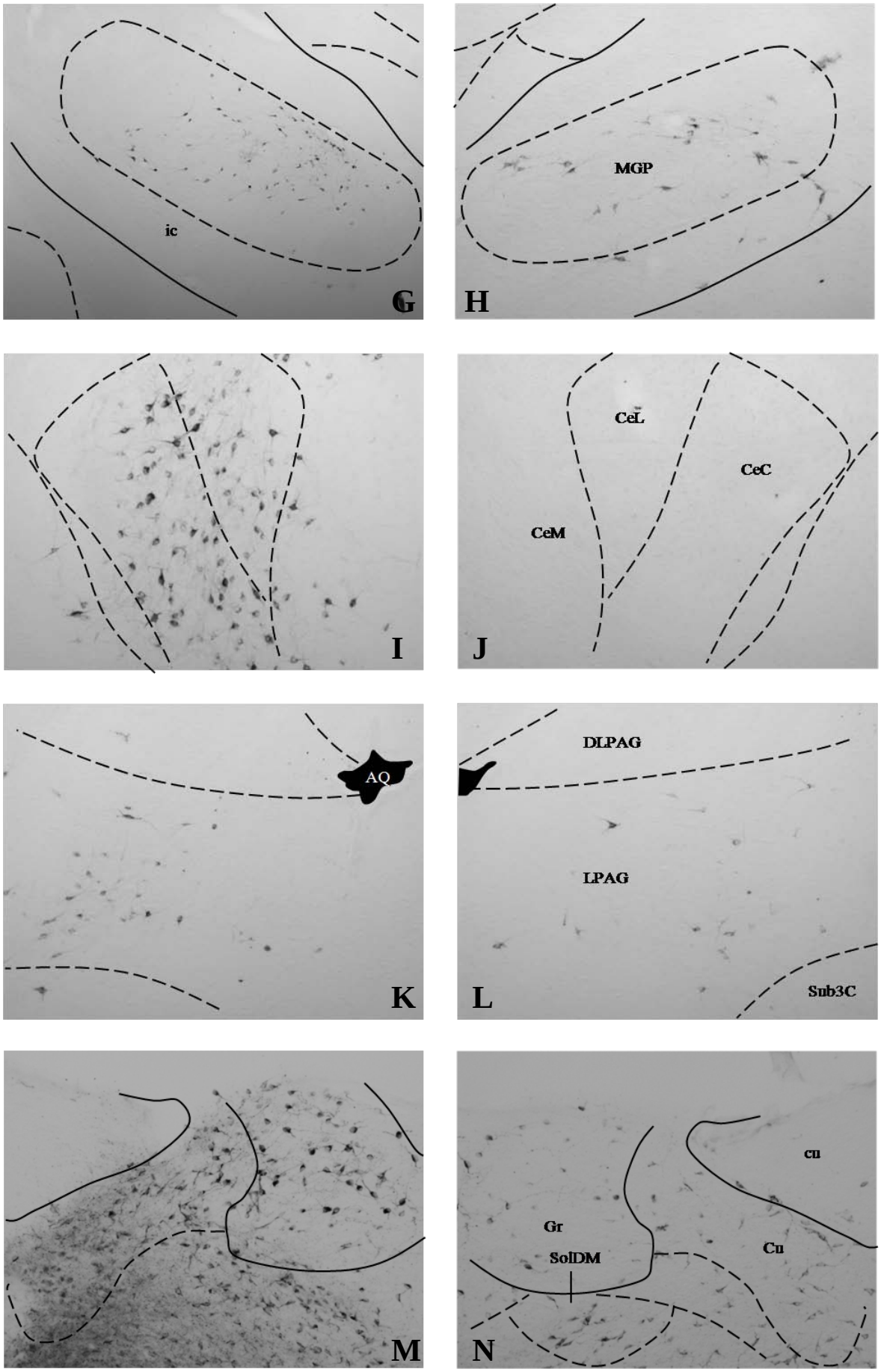
Figure 13 - Camera-lucida-like drawings (A–D) (and two photomicrographs) of four coronal brain sections presenting significant amount of BDA labelled fibers originated from the VRt. Note that in the black/gray area (scheme D) fibers have not been represented individually due to their high density resulting from the proximity to the injection site. RF, reticular formation; PB, parabrachial nucleus; RVM, rostroventral medulla; PAG, periaqueductal gray; PF, parafascicular nucleus.

3.2 – Retrograde Tracing Experiments

CTb-labelled neurons projecting to the VRt were found to be distributed throughout all the rostrocaudal extension of the VRt, mainly in the ipsilateral hemisection. A first analysis to the brain sections shows that at most caudal part, the higher number of neurons was

presented at areas of the brainstem, as PAG and DRt, which are known to be implicated in pain modulation. As to higher areas, such as PVN (Figures 14E, F) and MGP (Figures 14G, H) CTB-labelled neurons were mainly found ipsilateral to the injection site, despite a high labelling in the contralateral part. In subcortical telencephalic areas, a small number of labelled neurons was located in the ipsilateral bed nucleus of the stria terminalis (BST; Figure 14C), contrary to its contralateral part, where rare or none neurons appeared (Figure 14D). In the amygdala, a very strong projection was shown to occur, mainly along the entire rostrocaudal extent of the central amygdaloid nucleus (CeC), ipsilaterally (Figure 14I). Contralaterally, none CTb-labelled neurons were found (Figure 14J). In the motor cortex, a higher number of neurons were present in the DLO and in secondary (M2) motor cortice, mainly on the ipsilateral hemisphere (Figure 14A, B).

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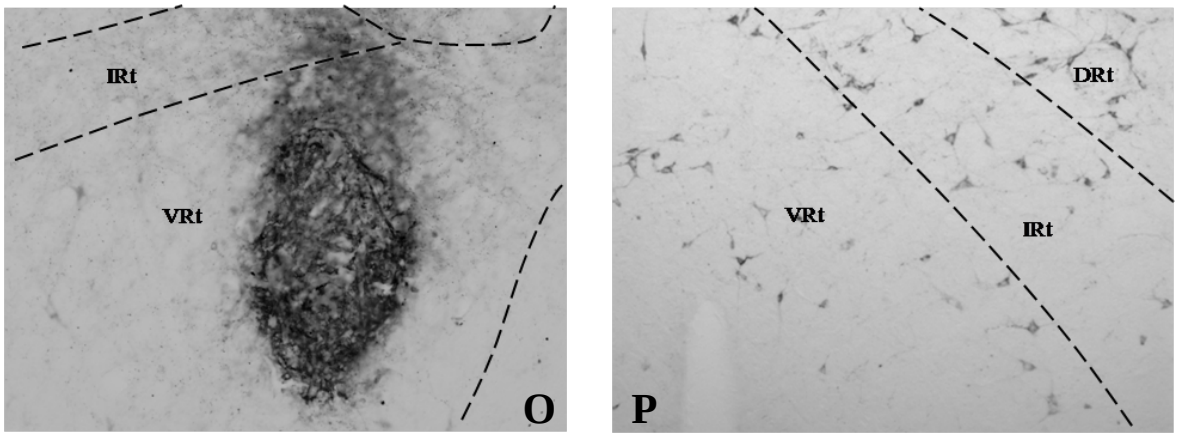

Figure 14 - Photomicrographs depicting retrogradely labeled cells in areas along the medulla oblongata, diencephalon and telencephalon, following CTb injections in the VRt. Panels at the left side are ipsilateral to the injection site, and contralateral at the right side. Scale bars = 200μM. All the panels are at the same magnification.

Chapter 4

DISCUSSION AND CONCLUSION

The present study maps the brain connections of the VRt by the use of anterograde and retrograde tracing with, respectively, BDA and CTb. The present results demonstrate that neurons from the VRt receive and project to areas of the brain involved in somatosensitive, emotional and cognitive pain processing. From all animals that received iontophoretic injections, only those cases with injections located within the nucleus with minimal involvement of adjacent areas were analysed for the present study.

The discussion will be organized into four parts: (1) a briefly approach to the tract-tracing methodology; (2) specificity of the VRt brain projection patterns; (3) a comparative study between the VRt and DRt and their integration in the medullary reticular formation; and (4) functional considerations concerning the main afferent connections of the VRt.

4.1 – Specificity of the Tract-tracing Methodology

It is known that axonal tracers are powerful tools for the study of neuronal circuits. Tract-tracing studies allow us to study the way in which two or more brain regions are connected. Three basic methods are used to apply the tracer material into the area of interest: pressure injection, iontophoretic injection and the mechanical insertion of dye crystals. Iontophoretic injections are commonly used in neurobiological experiments. This way of tracer application allow us to inject locally the tracer by the use of a glass micropipette with a small tip diameter (less than 20 JLm), and thus avoid the damage of fibers of passage. The tracer molecules are electrically charged, and therefore ejected from the micropipette by an applied electrical current created between the muscle of the rat and the tracer (for review see Köbbert et al., 2000). On the other hand, it is also important take into account the possibility of a deposit formation in the micropipette tip that can scratch the tissue and therefore influence the “reading” of the results. To evade this, low currents and, thus, long injections are advised (Kratskin et al., 1996).

In order to demonstrate axonal connections from the VRt, BDA was used as anterograde tracer. BDA has recently become the tracer of choice for the most anterograde studies, since it reveals excellent terminal morphology from small, localized injection sites, and for its large spectrum of survival time (3 days to 3 weeks) (Brandt and Apkarian, 1992; Veenman et al. 1992; Rajakumar et al. 1993; Reiner et al. 1993; Wouterlood and Jorritsma-Byham, 1993; Lanciego and Wouterlood 1994).. BDA is a very powerful tool for anterograde tract-tracing studies, and apparently more efficient than PHA-L, when injected

into the peripheral nervous system (Novikov, 2001). Knowing that BDA can be incorporated into injured dendrites and/or fibers at the injection site, small diameter micropipette tips were used during iontophoretic injections, thus avoiding the labelling of passing fibers. This was the case of the present study since virtually no labelled perikarya were found along the brain of injected rats.

The nuclei injected with BDA presented a small and delimited injection site with intense anterograde labelling. It was also observed a consistent efferent projection pattern along the rostral-caudal axis of the reticular formation mainly ipsilateral to the injection. Brainstem areas involved in pain modulation as the PB, the RVM and the PAG were also found to receive VRt projections. At the diencephalon, BDA labelling was sparser and mainly concentrated in the contra lateral thalamic parafascicular nucleus and in the LH.

As to the retrograde study, CTb has proven to be a very powerful tool for retrograde labeling of neurons, and therefore it has been used in several applications. Concerning the specificity of CTb as a retrograde tracer, several factors have been taken into account: Firstly, the placement of CTb in the VRt; big iontophoretic CTb injections resulted in diffuse injection sites, which seem to move dorsally towards to Gr and Cu. On the other hand, small iontophoretic injections resulted in restricted and perfectly defined injection sites, located within the boundaries of the VRt along the rostrocaudal extension of the nucleus. However, almost all of the small injections resulted in few or none labelling. Secondly, leakage of CTb: the micropipette track was observed after injection and no leakage of the tracer was detected, probably due to the waiting time between the end of injection and withdraw of the micropipette.. Thirdly, transneuronal transport: the idea that trans-synaptic transport accounted for the labelling of cells receiving projections from the VRt can be discarded, since CTb was shown not to be transported transneuronally after anterograde transport (Almeida et al., 1993). Lastly, the passing fibers: several studies have shown that CTb is not picked up by passing fibers (Lima and Coimbra, 1988; Tavares and Lima, 1994). Previous studies have shown that CTb injections into the spinothalamic tract didn’t cause spinal labelling (Lima et al., 1991), indicating that CTb is not picked up by passing fibers. In addition, it has been demonstrated that, with CTb, anterograde or retrograde tracing by passing fibers does not occur when injected iontophoretically (Luppi et al., 1990; Angelucci et al., 1996).

In table 2 are described some of the problems that we may encounter in studies using CTb and with them the possible reason and a possible solution (Conte et al., 2009).

Table 2 – CTb troubleshooting table.

Problem	Possible Reason	Solution
Labeling not visible or very dim	Tracer conjugates are incompatible with filter sets on the microscope	Ensure that all filter sets are compatible with the tracers. On our system, we have had good results using the FITC filter set for the AF 488 conjugate and the Texas red filter for the AF 594 conjugate (use a Cy3-equivilant filter for AF 555 and a Cy5-equivilant filter for AF 594). Also ensure that the UV light source is of good quality to provide enough light intensity for fluorescent labelling. If having problems capturing photos of the labelling, increase the exposure time. We typically use a 5-s exposure time using a Qimaging Retiga 4000R monochrome camera
	CTB solutions were vortexed	Vortexing CTB results in protein denaturation. When mixing the tracer, gently roll the vial to allow gradual saturation of the solid. If the tracer was vortexed, the injection site will typically look normal, but there will be little evidence of transport
	Improper injections	Ensure that the animal is positioned correctly in the stereotaxic device to make sure the coordinates are reliable. If there is a complete absence of injection sites (or they are very small and faint), then increase the injection volume or the tip diameter. Surgical damage to the brain tissue may also cause problems with the tracer transport. If no injection site is visible, then a clog in the pipette during injection is possible. To recognize and prevent clogs, observe the meniscus of the tracer in the pipette when injecting. If it does not move, then temporarily increase the injection duration/pressure until the clog is removed
	Improper storage of the tracer	The solid form of CTB is stable and can easily be stored at -20 °C for about 6 months. Always avoid repeated freeze–thaw cycles. Also ensure that the storage freezer is not equipped with an 'anti-frost' system, as this results in multiple freeze–thaw cycles (an easy workaround for this is to place the tracers in an insulated container with ice and place the container in the freezer). However, the solution form of CTB is less stable and more susceptible to contamination. Always ensure the solution is stored at 4 °C for no longer than a week. Although this solution could possibly be stored at -20 °C for an extended period of time, we advise against this. When we have used solutions in the past that had been stored at -20 °C, we experienced unreliable results. Furthermore, we also experienced significant necrosis at the injection site in some cases. We attribute these problems to possible crystallization of the tracer. If the solutions have to be stored at -20 °C, ensure that they are gradually defrosted
	Improper concentrations	A nonoptimal concentration of the tracer will most likely still show a normal injection site. However, labelling patterns may be very dim if the concentration is too low. We recommend a starting concentration of 1%. If this concentration does not work (assuming all other possible problems have been ruled out), increase the concentration to a maximum of 2%. For double injections, it is good to have different concentrations for each tracer (we typically find that the red conjugate is easier to visualize than the green conjugate). Note that increased background labelling when using higher concentrations has been experienced

High background fluorescence	Improper perfusion	If widespread non-neuronal cell labelling throughout the tissue that autofluoresce in all spectra is observed, an improper perfusion is likely to blame. In particular, increase the duration of the initial saline perfusion. Also ensure that the perfusion begins very quickly once the animal has been anesthetized before any clotting begins
Fading of cell labelling	Photobleaching as a result of prolonged exposure	Although Alexa Fluor is claimed to be more photostable than other fluorescent dyes, it is still susceptible to photobleaching when exposed to intense UV light for long periods of time. Ensure that exposure to light at all stages of the procedure (i.e., when the tracer is still a solution and when the sections are coverslipped) is minimized. We routinely mount two adjacent series of fluorescent sections to account for any possible fading: one for viewing the labelling patterns and the other for taking quality pictures
	Photobleaching due to prolonged storage	The AF dyes are typically more stable over long periods of time than other fluorescent dyes. Nevertheless, a slight fade in signal when viewing sections that are several months old is still noticed. To prevent any possible problems with the results, view samples and take pictures as soon as possible. Coverslipping the sections with Entellan (or other anti-fade medium) as quickly as possible also helps prevent fading
Anterograde labelling present	This is possibly due to a damaged injection site	Whenever necrosis within the injection site is observed, a degree of anterograde labelling is present in addition to retrograde labelling. To prevent injection necrosis, inject the tracer slowly. Typically, one should wait at least 10 s between each shot of tracer when using a picospritzer. Also, one should make sure that the pressure settings and duration of the picospritzer are not too intense. Using smaller pipette tips may also help prevent injection damage. Also, try to lower the concentration of the tracer, as high concentrations cause necrosis. Furthermore, ensure that the tracer is being stored properly. Typically, any damaged injection sites when given iontophoretic injections are not observed. Nevertheless, if necrosis is observed when using iontophoresis, double-check the injection parameters and ensure that the pipette tip is not too large. An often-overlooked factor that may cause a damaged injection site is the presence of air bubbles in the solution. Remove air bubbles by performing several test injections onto a piece of X-ray film when determining the picospritzer diameter

4.2 Specificity of the VRt Brain Projection Patterns

Several studies have demonstrated the ascending and descending projections of areas of the reticular formation in the rat (Fulwiler and Saper, 1984; Vertes and Kocsis, 1994; Halsell et al., 1996; Almeida et al., 2002; Cobos et al., 2003; Leite-Almeida et al., 2006).

As referred before, VRt is the caudal continuation of the nucleus reticularis gigantocellularis and continues caudally through the deep lamina of the spinal dorsal horn. It is located ventrally to the trigeminal subnucleus caudalis and the DRt. On the coronal

plane, the VRt can be delimited from the Cu dorsally, the Sp5C laterally, and dorsomedially, from Sol and DRt. Functionally, these areas can be separated individually by their particular afferent and efferent connectivity (for more detail see Leite-Almeida et al., 2006).

After BDA injections in the VRt, anterograde labelling was observed bilaterally in the caudal medulla, including the NTS and reticular formation areas such as the intermediate reticular nucleus (IRt), LRt, VLMlat and, to a lesser extend, the DRt. VRt neurons also projects to areas of the rostral medulla, namely to the RVM, despite in less number, to the pons, with labelling fibers mostly concentrated in the PB, KF and, to mesencephalic nuclei, with PAG as the major receptive area. The LH and PH hypothalamic nuclei and the VPL/VPH, Po and VM thalamic nuclei were the diencephalic areas to where the VRt projected more prominently. At the most rostral level, the VDB/HDB is the only telencephalic area that receives a significant amount of projections from the VRt. Areas strongly or moderately targeted by DRt efferents, such as 12, ECu, Gi, PCRt, Sp5, Me5, DR, VTA, Arc, LH, L/MPO, PC, Amy, BST, are not a powerful target of VRt neurons with rare, if not none, labelling fibers. To confirm these results, was done a search to articles about retrograde studies in those particular nuclei.

It is known that areas described above as receiving projections from the VRt have an important role in pain modulation, either in facilitation or inhibition. These include all relay areas of the descending hypothalamus–PAG–RVM–dorsal horn circuitry, the noradrenergic cell groups LC, A5 and A7, the NTS and nearly all brainstem areas of the reticular formation implicated in pain control. Like the DRt, projections to the medial thalamus and to the limbic system suggest an involvement of the VRt in the emotional processing of pain. To a better understanding, the limbic system is a collection of primitive brain structures located on top of the brainstem, under the cortex. Limbic system structures are known to be involved in many of our emotions and motivations, mainly those related to survival. Such emotions include fear, anger, and emotions related to sexual behaviour. The amygdala and the hippocampus are considered important areas of the limbic system that are involved in emotional and cognitive processing. Roughly speaking, the amygdala decides what memories must be stored and where, depending on how huge an emotional response an event invokes. The hippocampus sends memories out to the appropriate part of the cerebral hemisphere for long-term storage and retrieves them when

necessary. Its damage may result in an inability to form new memories. The diencephalon, which contains the thalamus and hypothalamus, is also included in the limbic system. The thalamus is involved in sensory perception and regulation of motor functions (i.e., movement), and is connected with areas of the cerebral cortex that are involved in sensory perception and movement with other parts of the brain and spinal cord that also have a role in sensation and movement. The hypothalamus, although a very small area, plays a very important role in regulating hormones, the pituitary gland, body temperature, the adrenal glands, and many other vital activities involved in the homeostasis of the body (Kandel et al., 1991).

On the other hand, projections to areas belonging to the extrapyramidal and orofacial motor system indicate an involvement in the motor reactions associated with pain. Taken together, these results came to reinforce what is already known about this nucleus as a medullary center of pain processing and modulation.

These results were supplemented with CTb injections into the nucleus. Preliminary data appointed to retrogradely labelled neurons in several areas along the rostrocaudal extension of the brain, mainly ipsilateral to the injection site. However, new CTb injections are required to confirm the results.

4.3 The VRt integrated is the medullary reticular formation – a comparative study with DRt

It is known that supraspinal pain control centers that are involved in the modulation of spinal nociceptive transmission can exert both an antinociceptive (inhibitory) and a pronociceptive (facilitating) role upon nociceptive spinal dorsal horn neurons (reviewed by Pertovaara, 2000; Lima and Almeida, 2002; Millan, 2002; Porreca et al., 2002; Gebhart, 2004; Leite-Almeida et al., 2006). Contrary to the VRt, the role of DRt in pain processing and modulation is well explored by anatomical, physiological and behavioural studies (for reviews see Villanueva et al., 1996; Lima and Almeida, 2002; Monconduit et al., 2002).

These two nuclei were recently shown to belong to the supraspinal pain control system. Neurons within the DRt are activated mostly by noxious stimulation converging from all the body and they were found to be involved in descending projection that will increases spinal nociceptive transmission and thus facilitating pain perception (Villanueva et al., 1988, 1989; Almeida et al., 1999). On the other hand, the activity of the neurons

found in the VRt, remains unaffected or is inhibited by noxious peripheral mechanical stimulation (Villanueva et al., 1988). Additionally, it has been reported that the VRt as an antinociceptive role, contrary to its dorsal part that is involved in the facilitation of nociception (Almeida et al., 1999).

As to the spinal circuitry, both nuclei are reciprocal connected with the spinal dorsal horn laminae implicated in nociception (Almeida et al., 1993; Tavares and Lima, 1994; Almeida et al., 1995, 2000; Villanueva et al., 1995; Raboisson et al., 1996; Almeida and Lima, 1997). Anatomical studies on the ascending and descending DRt pathways have shown the presence of a spino-DRt circuit made up of bidirectional connections between the dorsal DRt (DRtd) and laminae I and IV–VI, ipsilaterally, and the ventral DRt (DRtv) and laminae IV–VI, bilaterally (Lima and Almeida, 2002). As to the ascending spinal connections, the VRt receives afferents from laminae V and VII-X (Men6trey et al. 1983; Villanueva et al. 1991), whereas regarding the descending connections, VRt project mainly to the ventral horn (laminae VII-X), via the ventral funiculi and to laminae IV-V (Tavares and Lima, 1994; Villanueva et al., 1995).

The large spectrum of projections diverging and converging upon the DRt and VRt may explain the differences in the way that each one is involved in pain modulation. The brain connections of the DRt are extensive as revealed by several tracer studies (Almeida et al., 2002; Leite-Almeida et al., 2006). These data came to suggest that the DRt integrates information from the somatosensory, antinociceptive, autonomic, limbic, pyramidal and extrapyramidal systems while triggering its descending facilitating action upon the spinal nociceptive transmission (Almeida et al., 2006). As to the VRt, the main difference when compared with the DRt, is concerned with areas as 12, ECu, Gi, PCRt, Sp5, Me5, DR, VTA, Arc, LH, L/MPO, PC, Amy, BST, which were strongly or moderately targeted by DRt efferents, but constitute “weak” targets for VRt neurons.

4.4 Functional considerations

Sensorimotor and pain control systems

A large spectrum of brain areas belonging to the supraspinal pain control system project to the VRt and may modulate its activity. Therefore, areas previously shown as antinociceptive, such as the motor cortex, basal ganglia, amygdala, thalamus (PF, paraventricular thalamic nucleus, sensory thalamus), hypothalamus (LH, Pa, Arc, PH), the

PAG-RVM circuit, noradrenergic brainstem areas (LC/subcoeruleus, PBN, KF, A5, Sol, A1) and the brainstem reticular formation (DpMe, CnF, DRt, VLMlat) were shown here to project to the VRt. On the other hand, areas with both antinociceptive and pronociceptive actions, such as the RVM (Wei et al., 1999; Kovelowski et al., 2000) and Sol (Renet al., 1990; Ness et al., 2000), also project to the VRt. In addition, the contralateral and ipsilateral VRt itself have short-projecting neurons inside its borders. Altogether, these data suggest that the VRt may also contribute to mediate the balance between inhibiting and facilitating nociceptive actions that may, respectively, turn off or turn on the descending modulatory action of the supraspinal pain control system upon the spinal nociceptive transmission.

The VRt also receives strong projection from the motor cortex and from extrapyramidal areas, as substantia nigra, IO, PCRt and LRt, which may indicate its involvement in pain motor reaction. Adding to the fact that VRt projects to the spinal dorsal horn (Villanueva et al., 1995), it is possible that the VRt have some control on the motor reactions to noxious stimulation, and thus modulates the activity of spinal motoneurons.

Autonomic and limbic systems

The activity of VRt neurons can be influenced by the visceral motor system. The visceral (or autonomic) motor system is responsible to control involuntary functions mediated by the activity of smooth muscle fibers, cardiac muscle fibers, and glands, and is mainly controlled by parts of the brainstem and the hypothalamus (Loewy, 1990). The visceral motor system involves afferents from structures such as the dorsal motor nucleus of the vagus and the PrL and IL cortices. Other forebrain autonomic centres (reviewed by Loewy, 1990) that are involved in the organism homeostasis also projects to the VRt, as the BST, Ce, Pa and LH. Most of the above autonomic centres belong also to the limbic system (reviewed by Lopes-da-Silva et al., 1990), i.e. the prefrontal cortex (PrL, IL and OC), BST, Ce and SI. To the best of our knowledge, these data indicates that the VRT is not only involved in the transmission of ascending nociceptive information related to the motivational-affective component of pain, but also that its activity can be modulated by the reaction triggered by these dimensions of pain.

Chapter 5

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Chapter 6

ANNEXES

ANNEXE 1

Imunocitoquímica para revelar BDA

1º DIA – Corte, Recolha e Preservação dos Cortes Histológicos

1. Cortes recolhidos em PBS 0.1M, pH=7.2

2. Inibição da peroxidase endógena (330µL de H_2O_2 em 10mL de PBS 0.1M, pH=7.2) – 10 min

3. Lavagem em PBS 0.1M, pH=7.2 – 6x10 min

4. Incubação em Complexo Avidina-Biotina vector ELITE em PBS-T, pH=7.2, 1:200 (preparar com, pelo menos, 30 min de antecedência) – 1 hora

5. Lavagem em PBS-T 0.3%, pH=7.2 – 2x10 min

6. Lavagem em PBS 0.1M, pH=7.2 – 2x10 min

7. Lavagem em tampão TRIS-HCl 0.05M, pH=7.6 – 2x10 min

8. Reacção com DAB (10mg DAB + 20mL tampão Tris-HCl 0.05M, pH=7.6 + 4µL H_2O_2) – 20 min

9. Lavagem em tampão TRIS-HCl 0.05M, pH=7.6 – 2x10 min

10. Montagem dos cortes em lâminas de gelatina

11. Secagem na estufa a 37ºC

12. Passagem por xilol

13. Montagem com Eukitt

Reagentes

1. SORO FISIOLÓGICO A 0.9%

NaCl – 9g

Diluir em 1000mL de água destilada

2. PBS 0.02M, PH 7.2-7.4

$NaHPO_4.12H_2O$ – 14.34g
$NaH_2PO_4.H_2O$ – 1.31g
NaCl – 22.5g

Diluir em 2500mL de água destilada

3. TAMPÃO TRIS-HCL 0.1M, PH 7.6

TRIS – 12.2g
Água destilada – 500mL
HCl 0.1N – 750mL

4. ABC

Reagente A – 2 gotas
Reagente B – 2 gotas
Tampão fosfatos – 5mL

5. DAB

DAB – 10mg
TRIS – 20mL
H_2O_2 – 4µL

ANNEXE 2

Imunocitoquímica para revelar CTb

1º DIA – Corte, Recolha e Preservação dos Cortes Histológicos

1. Cortes recolhidos em PBS 0.1M, pH=7.2

2. Inibição da peroxidase endógena (330µL de H2O2 em 10mL de PBS 0.1M, pH=7.2) – 10 min

3. Lavagem em PBS 0.1M, pH=7.2 – 2x10 min

4. Lavagem em PBS-T 0.3%, pH=7.2 – 2x10 min

5. Incubação overnight em anti-CTb 1:40000 em PBS-T, pH=7.2 – mínimo de 8 horas

2º DIA – Reacções, Marcação e Montagem das Preparações

1. Lavagem em PBS-T 0.3%, pH=7.2 – 3x10 min

2. Incubação em anticorpo secundário biotinilado Horse anti-goat 1:200 em PBS-T, pH=7.2 – 1H

3. Lavagem em PBS-T pH=7.2 – 3x10 min

4. Incubação em Complexo Avidina-Biotina vector ELITE em PBS-T, pH=7.2, 1:200 (preparar com, pelo menos, 30 min de antecedência) – 1 hora

5. Lavagem em PBS-T 0.3%, pH=7.2 – 2x10 min

6. Lavagem em PBS 0.1M, pH=7.2 – 10 min

7. Lavagem em tampão TRIS-HCl 0.05M, pH=7.6 – 2x10 min

8. Reacção com DAB (10mg DAB + 20mL tampão Tris-HCl 0.05M, pH=7.6 + 4µL H2O2) – 20 min

9. Lavagem em TRIS-HCl 0.05M, pH=7.6 – 2x10 min

10. Montagem dos cortes em lâminas de gelatina

11. Secagem na estufa a 37ºC

12. Passagem por xilol

13. Montagem com Eukitt

ANNEXE 3

Coloração pelo Método de Tionina

Os cortes devem secar durante um período de 48h antes de se efectuar o procedimento. Todos os procedimentos à temperatura ambiente.

1. Mergulhar os cortes em acetona ácida (5 min).

2. Escorre e passar por água destilada.

3. Mergulhar os cortes na solução de tionina durante o tempo necessário de acordo com o grau de coloração pretendido (1 min para uma coloração forte).

4. Escorre e passar por água destilada. As lavagens devem ser tantas quantas as necessárias para uma completa remoção do corante.

5. Mergulhar os cortes em álcool ácido (1 min).

6. Escorre e passar por água destilada.

7. Deixar os cortes secarem, primeiro ao ar e depois na estufa a 37ºC.

Reagentes:

- Acetona (4 partes) + ácido acético glacial (1 parte); para um total de 250 mL = 200 mL + 50 mL

- Tionina 0,1% em formol* a 10%; juntar três gotas de ácido acético glacial por cada 100 mL.

* = formaldeído 37%

- Álcool ácido: álcool isopropílico* a 99% (2 partes) + ácido acético glacial a 10% (1 parte); para um total de 250 mL = 166mL + 83 mL.

* = 2-propanol ou isopropanol

ANNEXE 4

Perfusão, Fixação e Pós-Fixação

1. Prepara-se e coloca-se o fixador no sistema de fixação.

2. Anestesia-se o animal com hidrato de cloral a 35%.

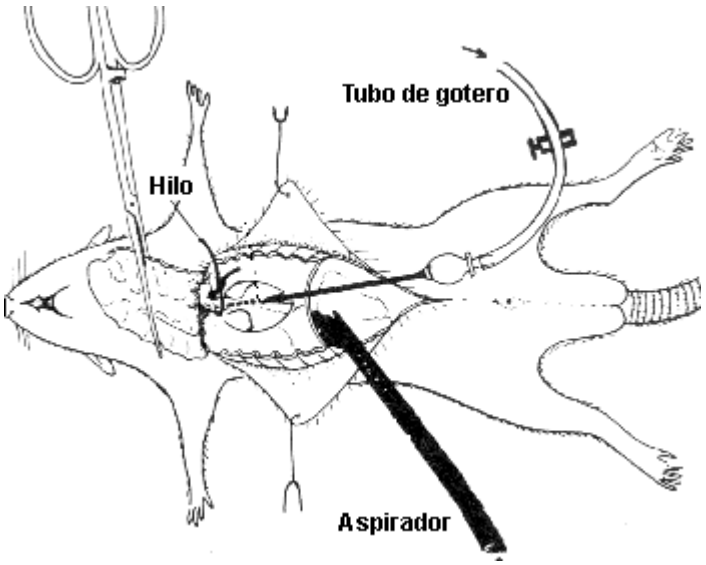
3. Abre-se a cavidade abdominal, expondo-se a base da caixa toráxica e o diafragma.

4. Corta-se o diafragma e as costelas, de cada lado dos pulmões, como indica a figura, deixando o coração descoberto.

5. Afasta-se o timo de forma a expor a aorta.

6. Lanceta-se o ventrículo esquerdo com tesoura fina e introduz-se a agulha até a aorta (vê-se a agulha à transparência).

7. Veda-se a agulha na aorta pinçando o ventrículo.



8. Caso seja necessário, corta-se o ventrículo direito e recolhem-se amostras de sangue, caso contrário, passa-se para o ponto 9.

9. Corta-se a aurícula direita e abre-se o sistema com PBS 0.1M, pH=7.2 até deixar de sair sangue.

10. Passa-se o fixador (paraformaldeído 4%) e fixa-se durante 30 min ou até terem corrido 500mL de fixador.

11. Depois de fixado, retira-se a camada muscular que rodeia o pescoço e coluna vertebral, com ajuda de um bisturi.

12. Descasca-se a parte cervical da coluna vertebral utilizando um forcep forte, expondo-se a medula espinhal e o bolbo raquidiano.

13. Descasca-se a calote craniana, expondo o cérebro que é retirado.

14. O material recolhido é colocado em paraformoldeído 4% durante 2 horas.

15. Sendo depois conservado até um máximo de 48h numa solução de sacarose:

- 30% se o corte for efectuado no criostato

- 8% se for cortado no vibratomo em PBS 0.1M, pH=7.2

Reagentes:

- Paraformaldeído a 4%

Paraformaldeído – 340g

Água destilada (ou PBS 0.1M)- 5950mL

NaOH – umas gotas

Diluir o paraformaldeído em 5950mL de água destilada (ou PBS) quase a ferver, até ficar límpido.

- Sacarose 8%

Sacarose – 80g

Tampão fosfatos 0.1M, pH=7.2 – 1000mL